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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 48/00, C12N 15/63, 15/11, C07K 14/00, C07H 21/04 — A1

(11) International Publication Number: **WO 99/36103**

(43) International Publication Date: 22 July 1999 (22.07.99)

(21) International Application Number: PCT/US99/00965

(22) International Filing Date: 15 January 1999 (15.01.99)

(30) Priority Data:
60/071,694 16 January 1998 (16.01.98) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 60/071,694 (CIP)
Filed on 16 January 1998 (16.01.98)

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(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: PREVENTION AND TREATMENT OF NEUROPATHY BY HEPATOCYTE GROWTH FACTOR

(57) Abstract

The invention features methods of preventing axonal degeneration and promoting axonal growth or axonal regeneration in neurons, by exposing the neurons to hepatocyte growth factor.

BNSDOCID: <WO___9936103A1_I_>

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PREVENTION AND TREATMENT OF NEUROPATHY BY HEPATOCYTE GROWTH FACTOR

Field of the Invention

This invention relates to the field of axonal regeneration in neurons.

Background of the Invention

Peripheral neuropathy (nerve dysfunction) resulting from degeneration or demyelination of peripheral nerve axons, may affect motor, sensory, or autonomic nerves. General clinical manifestations of peripheral neuropathy include alterations in sensation, alterations in autonomic functions (involving, for example, the heart, organs containing smooth muscle, and glands), and muscle weakness and atrophy.

For example, peripheral neuropathy is a common complication of diabetes mellitus, in which dysfunction of autonomic, sensory, and/or sensorimotor nerves results in an array of disabling symptoms including diarrhea, urinary retention, postural hypotension, and male erectile impotence. At its most extreme, autonomic dysfunction caused by peripheral neuropathy leads to cardiac arrhythmias and death.

Clearly, it would be desirable to prevent, retard, or reverse the disturbing and often life-threatening manifestations of peripheral neuropathy by stimulating growth or regeneration of peripheral nerve axons.

Neurons within the peripheral nervous system, including those affected by peripheral neuropathy, have the capacity for axonal growth and regeneration. Moreover, under certain conditions peripheral neurons are capable of significant axonal growth and regeneration following axonal injury. However, it is not understood what regulates such axonal growth and

regeneration.

Potential candidates for such regulators include autocrine growth factors. One multifunctional and widely-expressed cytokine, hepatocyte growth factor (HGF; see e.g. USPN 5,545,722, herein incorporated by reference), is known to function in an autocrine fashion to promote motility and transformation of epithelial cells. HGF mediates biological activity by binding to its receptor, the Met tyrosine kinase. Genetic ablation of either the HGF or Met receptor genes results in embryonic lethality by E14 due to deficits in the liver, placenta and migration of muscle progenitor cells.

Summary of the Invention

We have discovered a novel mechanism for promoting optimal axonal growth that involves localized hepatocyte growth factor (HGF). Localized exogenous HGF promotes the growth (but not survival) of sympathetic neurons. Moreover, sympathetic neurons co-express bioactive HGF and its cognate receptor, the Met receptor. Antibodies that inhibit HGF activity decrease neuron growth, but have no affect on survival. Hence, the administration of HGF may be used to promote axonal growth and regeneration for the prevention or treatment of neuropathies involving axonal degeneration, such as diabetic neuropathy.

In a first aspect, the invention features a method for promoting axonal growth or axonal regeneration of a post-natal neuron. The method includes administering an expression vector to the neuron, wherein the expression vector includes a hepatocyte growth factor gene operably linked to a promoter.

In a second aspect, the invention features a method for inhibiting axonal degeneration of a post-natal neuron. The method includes administering

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an expression vector to the neuron, wherein the expression vector includes a hepatocyte growth factor gene operably linked to a promoter.

In a third aspect, the invention features a method for treating or inhibiting neuropathy in a patient. The method includes administering a therapeutically effective dose of hepatocyte growth factor to the patient.

In a related fourth aspect, the invention features a method for treating or inhibiting neuropathy in a patient. The method includes administering an expression vector including a hepatocyte growth factor gene operably linked to a promoter to the patient.

In one embodiment of the third and fourth aspects of the invention, the patient is identified as having a neuropathy. In further embodiments, the neuropathy may be a symptomatic neuropathy or an asymptomatic neuropathy, and may be caused by axonal degeneration. Furthermore, in various embodiments the neuropathy may be an autonomic neuropathy, a sensory neuropathy, a sensorimotor neuropathy, or a motor neuropathy.

In other embodiments of the fourth aspect, the expression vector is expressed in a neuron, or is expressed in a non-neuronal cell in the region of the body where the neuropathy is present.

In various embodiments of the first, second and fourth aspects of the invention, the hepatocyte growth factor gene further encodes a signal sequence that directs secretion of hepatocyte growth factor from a neuron or from a non-neuronal cell, the expression vector may be an adenoviral vector, and the promotor may be a Tα1 α-tubulin promoter.

In other embodiments of the first through fourth aspects, the hepatocyte growth factor may comprise a non-cleavable sequence variant, and the hepatocyte growth factor or a hepatocyte growth factor expression vector is administered to a neuron selected from: a sympathetic neuron, a

parasympathetic neuron, a sensory neuron, or a motor neuron. In addition, the hepatocyte growth factor expression vector may be administered to the terminals of sympathetic neurons.

In still other embodiments of the third and fourth aspects of the invention, the patient is identified as at risk for diabetic neuropathy prior to said preventing, or the patient is identified as having diabetic neuropathy, or the patient is identified as having clinical manifestations of diabetic neuropathy prior to treatment. In still further embodiments the diabetic neuropathy may be caused by insulin-dependent diabetes or by non-insulin-dependent diabetes. The diabetic neuropathy may present as distal sensory polyneuropathy, sensorimotor polyneuropathy, autonomic neuropathy, visceral autonomic neuropathy, mononeuropathy, or mononeuropathy multiplex. Patients having such diabetic neuropathies may have symptoms including, but not limited to, foot ulcerations, cardiac arrhythmias, sexual impotence, chronic pain, or abnormal vascular responses as a result of their neuropathy.

By "hepatocyte growth factor" or "HGF" is meant a polypeptide that is substantially identical to an amino acid sequence set forth in Accession No. P14210 of the SWISS-PROT amino acid sequence database (http://www.expasy.ch/sprot or http://www.ebi.ac.uk/sprot). Included in the definition are HGFs from other species, such as mouse, rat, and chicken (see, e.g., SWISS-PROT Accession Nos. Q08048, P17945, and X84045), as well as naturally-occurring and artificially-produced amino acid sequence variants (such as those disclosed in "Single-Chain Hepatocyte Growth Factor Variants" (USPN 5,316,921) and "Hepatocyte Growth Factor Variants" (USPN 5,547,856)). HGF is capable of stimulating axonal growth and regeneration and inhibiting axonal degeneration of SCG neurons.

By "hepatocyte growth factor gene" or "HGF gene" is meant a

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segment of DNA that encodes hepatocyte growth factor, as defined above.

By "neuropathy" is meant a functional disturbance or pathological change in the peripheral nervous system. Motor, sensory, and autonomic functions may be equally or preferentially effected. Major clinical manifestations are muscle weakness and atrophy, alterations in sensory perception, and/or altered autonomic function. In extreme cases, neuropathy can lead to death. For example, the incidence of cardiac arrhythmia is increased in those suffering from autonomic neuropathy, and the incidence of fatal infection due to foot sores is increased in those suffering from sensory neuropathy.

Pathologically, a neuropathy may show mainly axonal degeneration, mainly segmental demyelination, or both. Neuropathies may be localized to one nerve (mononeuropathy) or to several individual nerves (mononeuropathy multiplex), or may be diffuse and symmetrical (polyneuropathy).

The etiology of a neuropathy may be known or unknown. Causes of neuropathy include metabolic disease (such as diabetes), poor nutrition (such as that seen in alcoholism), infection (such as that seen in infection by Herpes Zoster or Human Immunodeficiency Virus (HIV)), ischemia, toxin exposure, radiation, and inheritance of a genetic predisposition for developing a neuropathy.

By "in the region of the body where said neuropathy is present" is meant that vector-encoded HGF expressed by a cell within this specified region can diffuse to an affected axon and activate Met receptors on such axons within 24 hours of HGF synthesis.

By "transformation," "transfection," or "transduction" is meant any method for introducing foreign nucleic acid molecules into a cell. Lipofection, DEAE-dextran-mediated transfection, microinjection, protoplast fusion,

calcium phosphate precipitation, transduction (e.g., bacteriophage, adenoviral retroviral, or other viral delivery), electroporation, and biolistic transformation are just a few of the methods known to those skilled in the art which may be used.

By "transformed cell," "transfected cell," or "transduced cell," means a cell (or a descendent of a cell) into which a DNA molecule encoding a polypeptide of the invention has been introduced, by means of recombinant DNA techniques.

By "promoter" is meant a minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell type-specific, tissue-specific, temporal-specific, or inducible by external signals or agents; such elements may be located in the 5' or 3' or intron sequence regions of the native gene.

By "operably linked" is meant that a gene and one or more regulatory sequences are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequences.

By "expression vector" is meant a genetically engineered plasmid or virus, derived from, for example, a bacteriophage, adenovirus, retrovirus, poxvirus, herpesvirus, or artificial chromosome, that is used to transfer an HGF coding sequence, operably linked to a promoter, into a host cell, such that the encoded HGF is expressed within the host cell.

By "degeneration" is meant that the length of an axon or the density of axons in the peripheral nervous system (PNS) decreases as a result of neuropathy. The relative level of degeneration may be assessed using methods known in the art for determining relative PNS function in a patient. The

degeneration is by at least 5% (compared to a normal subject not experiencing neuropathy), preferably by at least 15%, more preferably by at least 25%, more preferably by at least 35%, and most preferably by at least 45%.

By "growth" or "regeneration" is meant that the length of an axon or the density of axons in the PNS, particularly in a PNS affected by neuropathy, increases as a result of a method of the invention. The growth or regeneration may be measured by methods known in the art for assessing the relative function of a subject's PNS.

By "inhibiting" is meant administering HGF or an HGF expression vector in order to delay the onset of neuropathy or axonal degeneration in a patient at risk therefor or to decrease the severity of the impending neuropathy or axonal degeneration in such a patient. The delay is by at least one day, preferably by at least one week, more preferably by at least one month, still more preferably by at least six months, and most preferably by at least one year. The decrease is by at least 10%, more preferably by at least 25%, still more preferably by at least 40%, yet more preferably by at least 60%, and most preferably by at least 70%.

By "treating" is meant administering HGF or an HGF expression vector in order to delay the worsening of an already-present neuropathy or to improve the clinical status of a patient with a neuropathy. Preferably the delay is by at least one month, more preferably by at least three months, still more preferably by at least six months, and most preferably by at least a year. The improvement is by at least 10%, more preferably by at least 25%, still more preferably by at least 40%, yet more preferably by at least 60%, and most preferably by at least 70%. The relative efficacy of the treatment may be assessed using methods known in the art for evaluating the relative function of a patient's PNS.

By "therapeutically effective dose" is meant an amount of hepatocyte growth factor given to a patient for treating or inhibiting a neuropathy as defined above. Such a dose is typically in the range of about 1 μg/kg to1 mg/kg of body weight.

By "signal sequence" is meant an amino acid sequence at the amino terminus of a protein, that, when present, direct secretion of the protein from the cell. One example of a signal sequence is amino acids 1-31 of amino acid sequence of the hepatocyte growth factor precursor set forth in Accession No. P14210 of the SWISS-PROT amino acid sequence database. Alternatively, an artificial signal sequence or signal sequence from another secreted protein may be fused at the amino terminus of mature hepatocyte growth factor (e.g., amino acids 32-728 of SWISS-PROT Accession No. P14210), or biologically active (e.g., stimulating axonal growth or regeneration or inhibiting axonal degeneration) fragments thereof.

By "identity" is meant that a polypeptide sequence possesses the same amino acid residue at a given position, compared to a reference polypeptide sequence to which the first sequence is aligned. Sequence identity is typically measured using sequence analysis software with the default parameters specified therein, such as the introduction of gaps to achieve an optimal alignment (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705).

By "substantially identical" is meant a polypeptide exhibiting at least 75%, preferably at least 85%, more preferably at least 90%, and most preferably at least 95% identity to a reference amino acid. The length of comparison sequences is at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino

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acids.

By "terminal of a sympathetic neuron" is meant the region of the neuronal axon that is furthest from the neuronal cell body.

Brief Description of the Drawings

Figs. 1A-1B are representations of PCR assays showing that Met (Fig. 1A) and HGF (Fig. 1B) are expressed in SCG sympathetic neurons *in vitro* and *in vivo*.

Fig. 1C is a representation of a Western blot showing that Met is expressed in SCG sympathetic neurons *in vitro* and *in vivo*.

Figs. 2A-2B are representations of *in situ* hybridization assays showing that Met (Fig. 2A) and HGF (Fig. 2B) are expressed in adult mouse SCG neurons.

Fig. 2C is a representation of an immunohistochemistry assay showing that Met is expressed in adult mouse SCG neurons.

Fig. 2D is a representation of an immunofluorescence assay showing that Met is expressed in cultured neonatal sympathetic neurons.

Figs. 2E-2F are representations of photomicrographs showing MDCK cells that were allowed to cluster, after which they were exposed to control medium (Fig. 2E) or medium conditioned by cultured neonatal SCG sympathetic neurons (Fig. 2F).

Figs. 3A-3D are representations of photomicrographs showing that treatment with HGF induces c-fos expression in cultured neonatal SCG sympathetic neurons.

Figs. 4A-4F are graphs showing that exogenous HGF promotes growth but not survival of sympathetic neurons, and that endogenous HGF in sympathetic neurons is necessary for optimal neuronal growth but not for

survival.

Figs. 5A-5F are representations of phase contrast photomicrographs showing that endogenous HGF is necessary for optimal growth of SCG neurons.

Figs. 6A-6G are graphs showing growth of sympathetic neurons in compartmented cultures in the presence of HGF or anti-HGF antibody.

Figs. 7A-7B are representations of phase contrast photomicrographs showing that endogenous HGF is necessary for optimal growth of cultured sympathetic neurons in compartmented cultures.

Figs. 7C is a representation of a Western blot for detecting α-tubulin in lysates of axons in side compartments of compartmented cultures.

Detailed Description of the Invention

The cellular mechanisms that regulate axonal growth during development or axonal regeneration are not well-understood. In this regard, the studies reported here identify a novel mechanism in sympathetic neurons: autocrine HGF provides an intrinsic local "motor" for promoting axonal growth without affecting neuronal survival. The autocrine nature of this local "motor" makes it uniquely suited to drive axonal growth during periods when extrinsic sources of growth factors are few, such as during developmental axon extension and/or following axonal injury. Specifically, our experiments support the following conclusions.

First, sympathetic neurons coexpress the Met receptor and HGF, both *in vivo* and in culture. This Met receptor is functional and is distributed on both neurites and cell bodies, and the HGF is bioactive.

Second, exogenous HGF does not support survival of sympathetic neurons, but leads to robust neuronal growth, at least partially by locally

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activating axonal Met receptors. In the presence of low levels of NGF, addition of HGF increases neuritic density to an extent similar to higher levels of NGF. HGF does not, however, require NGF; when neuronal survival is maintained by KCl (which does not itself promote growth (Franklin et al., *J. Neurosci.* 15:643-664, 1995) exogenous HGF robustly increases neurite density, and, to a lesser extent, the rate of forward axonal growth.

Third, and most importantly, neutralization of endogenous HGF with function-blocking antibodies does not affect sympathetic neuron survival, but markedly decreases growth. The decrease in neuritic density is comparable to the decrease observed when neurons are switched from NGF to KCl, while there is lesser, but highly significant effect on forward extension rate.

Fourth, the effect of HGF on neuronal growth is substrate-independent, and is at least partially mediated locally: neutralization of axonal HGF decreases the rate and density of axonal growth, whereas neutralization of HGF in cell bodies and proximal neurites does not affect the rate of extension of distal axons.

Together, these data support the conclusion that HGF secreted by axons interacts locally with axonal Met receptors to increase the rate of axonal growth. This novel local autocrine loop has important implications for neuronal growth both during development and following axonal injury. In particular, HGF is likely to be useful in the treatment of medical conditions in which promotion of axon growth or regeneration is desirable. Such conditions include, but are not limited to, nerve damage caused by trauma or neuropathy. Examples of neuropathies that are likely to be prevented or ameliorated by HGF include neuropathies caused by metabolic diseases (such as diabetes), poor nutrition (such as alcoholic neuropathy), infection, ischemia, toxins, and radiation.

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Moreover, an autocrine axonal HGF loop does not preclude additive effects with any HGF encountered in the path of the growing axons. As shown here, sympathetic axons can still respond to exogenous HGF, even in the presence of an autocrine axonal loop.

Administration of HGF Polypeptides and HGF Expression Vectors

An HGF protein or gene may be administered within a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer HGF polypetides or genes to patients suffering from neuropathies or other injuries involving axonal degeneration. Administration may begin before the patient is symptomatic. Any appropriate route of administration may be employed, for example, administration may be parenteral, intravenous, intra-arterial, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, by suppositories, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found, for example, in *Remington's Pharmaceutical Sciences*, (18th edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, PA. Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene

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copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for HGF modulatory compounds include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

Expression vectors encoding HGF also may be introduced into cells by transfection. Lipofection, DEAE-dextran-mediated transfection, microinjection, protoplast fusion, calcium phosphate precipitation, adenoviral delivery, retroviral delivery, electroporation, and biolistic transformation are just a few of the methods known to those skilled in the art which may be used.

The following examples are to illustrate the invention. They are not meant to limit the invention in any way.

Example I: General Methods

Mass Cultures of Sympathetic Neurons

Mass cultures of pure sympathetic neurons from the SCG of postnatal day 1 rats (Sprague-Dawley rats supplied by Charles River Breeding Laboratories, Quebec, Canada) were prepared and cultured either in L15 media as previously described (Ma et al., *J. Cell. Biol.* 117:135-141, 1992; Belliveau et al., *J. Cell Biol.* 136:375-388, 1997) or in UltraCulture (BioWhittaker, Walkersville, MD), a defined medium containing 2 mM glutamine and 1% penicillin/streptomycin. No differences were observed in experimental results obtained in the two types of media. Neurons were plated on rat tail collagen-coated tissue culture dishes: 6-well plates (Falcon Labware, Becton Dickinson & Co., Lincoln Park, NJ) for biochemistry, and 48-well plates for

survival assays. Low density SCG cultures for neurite extension assays were plated on 24 well dishes coated with rat tail collagen.

For survival assays, NGF-depcndent neurons were selected by culturing sympathetic neurons for 5 days in the presence of 50 ng/ml NGF, as previously described (Ma et al., *supra*; Belliveau et al., *supra*). Neurons were washed three times for 1 hour each in neurotrophin-free media, and were then fed with media containing various concentrations of NGF plus HGF, NGF plus anti-HGF, or HGF alone. Analysis of survival was performed 48 h later by using nonradioactive cell proliferation (MTT) assays (CellTitre 96, Promega, Madison, WI; Belliveau et al., *supra*). Fifty µl of the MTT reagent was added to 500 µl media in each well and left for 2 hours at 37°C. After aspiration of the MTT-containing media, 100 µl of a 0.065N HCl/isopropanol mixture was added to each well to lyse the cells. Colorimetric analysis was performed using an ELISA reader. Each condition was repeated in triplicate. Zero ng/ml NGF was considered 0% survival, and 10 ng/ml was considered 100% survival. All other conditions were related to these values.

For neurite extension assays, neurons were cultured in 10 ng/ml NGF for 1 or 4 days. Neurons were then switched into media containing 10 ng/ml NGF plus HGF, or 10 ng/ml NGF plus anti-HGF antibody. After 2 additional days in culture, neurons were photographed and the number of neurite intersections were determined as described previously (Belliveau et al., *supra*). Briefly, regions in sister cultures containing a similar number of neuronal cell bodies were sampled and photographed, all interceptions and bifurcations of neurites within these windows were counted, and the number of intersections were normalized to the number of cell bodies. At least four windows were analyzed for each sample. Results are expressed as the mean density plus or minus the standard error of the mean. Statistical comparison was performed

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using Student's T-test.

For the KCl experiments, mass cultures of neonatal sympathetic neurons were cultured as described above at a density of approximately one ganglion per well of a four-well plate. Four days following plating, cultures were washed 4 times, 1 hour each, with serum- and NGF-free medium. Cultures were then switched to medium containing 50 mM KCl with or without added growth factors. Two days following the switch, three independent culture wells were photographed (five photographs per well) and the neurite process density determined as described above.

Two sources of HGF were used for these experiments; HGF purified from the conditioned medium of COS cells transfected with human HGF cDNA by HPLC (purified hHGF) (Zhu et al., *Cell Growth Diff.* 5:359-366, 1994), and recombinant human HGF kindly provided by Genentech (rhHGF). For purified hHGF, quantitation of activity was performed using scatter assays (see below, Stoker et al., *Nature* 327:239-242, 1987); the lowest amount of purified hHGF that caused MDCK cells to scatter was considered to be 1 unit. Two previously-characterized, function-blocking HGF antibodies were also used; a sheep anti-rhHGF antiserum provided by Genentech Inc. (the kind gift of Dr. Ralph Schmall; Tsao et al., *Cell Growth Differ.* 4:571-579, 1993), or purified goat anti-human HGF IgG purchased from Sigma (Rubin et al., *Proc. Natl. Acad. Sci. USA* 88:415-419, 1991).

Compartmented Cultures of Sympathetic Neurons

Compartmented cultures of pure sympathetic neurons were established according to previously described procedures (Campenot, "Compartmented culture analysis of nerve growth." In: *Cell-Cell Interactions: A Practical Approach.* Stevenson et al. Eds. Oxford Univ. Press, Oxford pp

275-298, 1992; Toma et al., *Dev. Biol.* 184:1-9, 1997). Briefly, SCGs were removed from postnatal day 1 rats. The ganglia were subjected to a combined trypsin and mechanical dissociation procedure, and plated into compartmented dishes. The compartmented dishes were constructed from collagen-coated 35 mm Falcon tissue culture dishes in which 20 parallel collagen tracks had been formed on the dish by scraping the dried collagen from the dish surface with a pin rake (Tyler Research Instruments, Edmonton, AB, Canada). In some experiments, the dishes were coated with poly-D-lysine and laminin, and the tracks were formed in the same manner. The scratched region of the dish was then wetted with culture medium, and a Teflon divider (Tyler Research Instruments, Edmonton, AB, Canada) that partitioned the dish into three compartments was sealed to the dish floor with silicone grease. Dissociated sympathetic neurons were plated in the center compartment at a density of about 1.5 ganglia per dish as previously described, and within 1-2 days neurites had entered the left and right compartments. Culture medium was UltraCulture (BioWhittaker, Walkersville, MD) supplemented with 2 mM L-Glutamine, 1% Penicillin/Streptomycin (BioWhittaker) and 0.4% Methylcellulose (Sigma Chemical Co., St. Louis, MO). Unless otherwise indicated, 3% rat serum (Harlan, Indianapolis, IN) was added only to the center compartments which contained the cell bodies and proximal neurites. Nonneuronal cells were eliminated using 10 μM cytosine arabinoside (Sigma Chemical Co., St. Louis, MO) in the central compartment during the first 4-5 days in culture. All three compartments were supplied with 2.5S NGF (Cedarlane Laboratories LTD, Hornby, ON, Canada), as described below.

To examine the effects of HGF or function-blocking HGF antibodies on axon extension in the side compartments, neurons were plated in the central compartment in the above medium supplemented with 10 ng/ml NGF. On day

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zero of culturing, the right compartment received either 1 or 3 ng/ml NGF plus 30 ng/ml HGF (Genentech), or 10 ng/ml NGF plus 5 µl/ml anti-rhHGF antiserum (Genentech). The left compartment of each culture received either 1, 3 or 10 ng/ml NGF with or without the addition of 5 µl/ml nonimmune sheep serum (Sigma Chemical Co.) and served as a control. Alternatively, anti-rhHGF was added to the center compartment. Within 1-2 days neurites crossed the silicone grease barriers and entered the side compartments in all cultures. Culture medium was routinely changed every 3-4 days. Neurite extension along each track in the right and left compartments of each culture was measured by an ocular microMeter using an inverted phase-contrast microscope (Axiovert 100, Carl Ziess). Neurite extension was measured at timepoints ranging from 2 to 7 days. Results are expressed as mean neurite extension plus or minus the standard error of the mean, and statistical analysis was performed using Student's T-test.

For the KCl experiments, compartmented cultures of neonatal sympathetic neurons were established as above, and after 4 days cultures were washed 4 times for 1 hour each, with serum- and neurotrophin-free medium. Following these washes, cell bodies and proximal neurites were switched to media containing 50 mM KCl, while the side compartments were switched to the same medium with or without 30 ng/ml HGF.

Scatter Assays

For detection of bioactive HGF, neurons were cultured for 4 days in 10 ng/ml NGF, followed by three washes with neurotrophin-free media for 1 hour each. Neurons were then switched to the same media plus 10 ng/ml NGF, and conditioned media was collected 8 or 24 hours later. For the scatter assay, MDCK cells were cultured in DMEM medium and plated at a density of 2 x

104 cells in 24 well dish, and left to settle overnight. MDCK cells were then switched to DMEM medium containing a 1:100 dilution of the sympathetic neuron conditioned-media, or unconditioned medium (with or without 10 ng/ml of NGF), and left for 8 or 24 hours. Scatter activity was analyzed as previously described (Stoker et al., *Nature* 327:239-242, 1987).

***In Situ* Hybridization**

SCG were dissected from adult CD1 mice, fixed in 4% paraformaldehyde in PBS for 30 minutes, and cryoprotected in graded sucroses (12%, 16% and 18%). Ten-μm cryostat sections were cut, mounted on Superfrost slides (Fisher), briefly air-dried, fixed in 4% paraformaldehyde in PBS for 5 min at room temperature, and washed twice in PBS. For *in situ* hybridization, slides were treated with proteinase K (1 μg/ml) in 0.1M Tris-HCl pH 7.5, 50 mM EDTA, and 2 mM CaCl2 at 37°C for 10 min, followed by incubation in 0.1M Triethanolamine containing 0.25% acetic anhydride for 10 min. Slides were washed in 3X PBS for 5 min, followed by three washes with 2X SSC for 5 min each, then prehybridized in a buffer containing 50% deionized formamide, 5X SSC, 5X Denhart's solution, 250 mg/ml tRNA and 200 mg/ml salmon sperm DNA at RT for at least 1h. Sections were hybridized in the same solution plus 5 ng/ml digoxigenin-labelled probes at 45°C overnight. Slides were then washed once with 2X SSC for 20 min, treated with 25 ug/ml RNAse in 0.1 M Tris plus 150 mM NaCl for 30 min at 37°C twice, followed by washing twice with 0.2X SSC and twice with 0.1X SSC at 55°C for 15 min each, and then blocked with 2% normal sheep serum and 0.3% Triton X-100 in Buffer 1 (100 mM Tris-HCl. pH 7.5, 150 mM NaCl) for 1 hour. To detect specific hybrids, slides were then incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (1 to 1,000

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dilution in Buffer 1) for 30 min, then washed twice (15 min each) with Buffer 1 and rinsed in Buffer 3 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$). The hybrids bound to anti-digoxigenin antibody were visualized by the color reaction with 337.5 µg/ml nitrobluetetrazolium salt (NBT), 175 µg/ml 5-bromo-4-chloro-3-indolyl-phosphate and 0.24 µg/ml Levamisole in Buffer 3, and colour was allowed to develop overnight in the dark. The reaction was terminated by incubation with 100 mM Tris-HCl, pH 8.0, 1 mM EDTA for 5 min. Slides were dehydrated, incubated in xylene, mounted with Permount and stored at 4°C in the dark. Slides were viewed and photographed on a light microscope.

The probe used for Met *in situ* hybridization corresponded to nucleotides 434-886 of the murine cDNA (Yang et al., *Development* 122:2163-71, 1996). HGF antisense probes were used as described in Sonnenberg et al., *J. Cell Biol.* 123:223-235, 1993. Nonradioactive antisense and sense riboprobes were synthesized by in vitro transcription using digoxigenin-UTP following the manufacturer's instructions (Boehringer Mannheim).

Immunocytochemistry

Cryosections of adult SCG were prepared as described for *in situ* hybridization. Sympathetic neurons were plated on poly-D-lysine plus laminin coated cover slips, maintained for 4 days in 10 ng/ml of NGF and then fixed in acetone and methanol (1:1 V/V) for 5 min at RT, and allowed to air dry. Sections (on slides prepared as above) or sympathetic neurons (on coverslips) were blocked with 4% goat serum plus 4% rat serum in PBS supplemented with 0.1% Tween-20 (PBST) for 1 hour, incubated with an anti-Met peptide antibody (1:150) (Yang and Park , *Dev. Biol.* 157:308-320, 1993) with or

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without 10 μg of competed peptide at 4°C overnight and then washed three times for 15 min each with PBST. To visualize the primary antibody, sympathetic neurons on cover slips were incubated with CY3-conjugated goat anti-rabbit IgG antibody (1:2000) (Jackson Immunolabs), washed three times for 15 min each with PBST, and mounted using Sigma mounting medium. Tissue sections were incubated with biotinylated anti-rabbit IgG, and the ABC visualization kit (Vector) was used according to the manufacturer's instructions.

RNA Extraction and Reverse Transcriptase PCR Amplification

Tissues (including SCGs) were dissected from adult CD1 mice, and total RNA was prepared following the protocol of Chomczynski and Sacchi, *Anal. Biochem.* 62:156-159, 1987. cDNA was synthesized from 5 μg of total RNA using the cDNA synthesis kit from Gibco BRL following the manufacturer's instructions. To amplify an HGF-specific product, two oligonucleotide primers P1 (5'-484 CCATGAATTTGACCTCTATG 503-3') and P2 (5'-760 ACTGAGGAA-TGTCACAGACT 741-3') were selected. Two specific oligoncleotide primers were also used to detect Met specific product, P3 (5' -272 AGATGAACGTGAACATGAAG 291-3'), P4 (5' -566 CTAATGAGTTGATCATCATAG 546-3'). The PCR reaction contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 200 mM dNTP, 10 pM of 5' and 3' HGF oligonucleotide primers, 2 μl cDNA template and 1 U Taq polymerase (BRL). Amplification was performed for 45 cycles: 94°C (1 min), 48°C (2 min), 72°C (2 min) in a Perkin Elmer Cetus DNA thermal cycler. The PCR products were separated on a 1.5% agarose gel and transferred to a Hybond N membrane (Nycomed Amersham plc, Buckinghamshire, UK). An internal HGF (P5, 5'-656

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ACCTACAGGAAAACTACTG 675-3') or Met oligonucleotide (P6, 5' -487 TGGCTTTGCTGCTGCAGTC 469-3') (100 ng) was end-labelled using T4 polynucleotide kinase. The membrane was prehybridized in 6X SSC, 1% SDS, 5X Denhardt's solution and 200 μg/ml salmon sperm DNA at 42°C for 1 h and then hybridized in the same solution plus 50% formamide and 1 X 10^6 cpm/ml of labelled oligonucleotide probe at 42°C overnight. The membrane was washed with 2X SSC and 0.1% SDS for 15 min and then exposed to X-ray film.

Immunoprecipitations and Western Blot Analysis

Primary neonatal sympathetic neurons (Sym neurons) cultured for 4 days, postnatal day 1 (P1) SCGs, or compartmented cultures of sympathetic neurons were lysed in cold Tris buffered saline (TBS) containing 137 mM NaCl, 20 mM Tris (pH 8.0), 1% (v/v) NP-40, 10 % (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 0.2 mg/ml leupeptin, 5 mM phenanthroline, and 1.5 mM sodium vanadate. The lysates were normalized for protein concentration using a BCA Protein Assay Reagent (Pierce, Rockford, Ill). For analysis of Met, 1.25 mg protein was immunoprecipitated with 10 μl anti-Met peptide antibody for 3 hours at 4°C (Yang and Park , *Dev. Biol.* 157:308-320, 1993), then incubated with Protein A-sepharose (Pharmacia and Upjohn, Kalamazoo, MI) for 1.5 hours at 4°C followed by centrifugation. Alternatively, 1.25 mg protein was precipitated with wheat germ lectin-agarose (WGA) (Pharmacia) for 3 hours. In both cases, the precipitate was washed three times with cold lysis buffer, proteins were separated on an 8% SDS-PAGE gel, and then transferred to 0.2 μm nitrocellulose for 1.0 hours at 0.5 amps. Transferred membranes were then washed 2 X 10 min in TBS, blocked in 2% BSA (Sigma Chemical Co., St.

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Louis, MO) in TBS for 2.5 hours, washed 2 X 10 min in TBS, and incubated overnight at 4°C with the anti-Mct-peptide antibody (1:150). Secondary antibodies were incubated for 1.5 hours at room temperature, and were used at dilution of 1:2000 for Protein A-HRP (Sigma Chemical Co). Detection was carried out using enhanced chemiluminescence (Nycomed Amersham plc, Buckinghamshire, UK) and XAR X-ray film (Kodak, Rochester, NY).

Analysis of tubulin in the neurites of sympathetic neurons in compartmented cultures was performed by Western blot analysis. Compartmented cultures were established on a poly-D-lysine and laminin substratum with 10 ng/ml NGF in all compartments, and 5 µl/ml anti-HGF in one side compartment. Six days following establishment of the cultures, the neurites from each side compartment were lysed in cold Tris-buffered saline lysis buffer as described above except that sodium dodecyl sulphate was added to a final concentration of 0.1%. For analysis of tubulin levels, 20 µg of total protein from each treatment group was separated by SDS-PAGE on a 7.5% gel, and transferred to 0.2 µm nitrocellulose membrane. The transferred membranes were then washed in TBS and blocked in 2% BSA, as described above. The membranes were incubated at 4°C with an α-tubulin monoclonal antibody (Cedarlane Laboratories LTD, Hornby, ON, Canada) at a concentration of 0.05 µg/ml. Detection was carried out using enhanced chemiluminescence (Amersham) and XAR X-ray film (Kodak, Rochester, NY).

c-Fos Stimulation

Acutely dissociated sympathetic neurons from postnatal day 1 rat SCG were plated on poly-D-lysine and laminin coated cover slips in L15-CO_2 medium without NGF for three hours (Wyatt and Davies, *J. Cell Biol.*

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130:1435-46, 1995). Neurons were then cultured with media containing various amount of HGF or NGF respectively for 3 hours. Cells were fixed in acetone and methanol (1:1 v/v), blocked with 2% goat serum in PBST, and then incubated with anti-fos antibody (1:50) (Oncogene Science, Uniondale, NY) overnight at 4°C. After 3 X 15 min washes with PBST, cells were incubated with biotinylated anti-mouse IgG, and the antibody reaction was detected using the ABC kit. For the function-blocking antibody experiments, HGF was preincubated with an excess amount of anti-HGF antibody at 4°C for three hours, and then added to the culture medium for three hours.

Example II: HGF and its receptor, the Met tyrosine kinase, are coexpressed in sympathetic neurons in vivo and in culture

To determine whether HGF and/or its receptor, the Met tyrosine kinase, are expressed in sympathetic neurons, we first examined the mouse superior cervical ganglion (SCG) by RT-PCR (Figs. 1A and 1B). Total RNA was isolated from the neonatal and adult SCG, from cultured neonatal sympathetic neurons (Sym neurons) and, for comparison, from the adult brain and liver, both of which are known to express HGF and Met mRNAs. RT-PCR analysis revealed that HGF and Met mRNAs were expressed both in the newborn and in the adult SCG (Figs. 1A and 1B; the (-) in the last lanes indicates that no cDNA was added to the PCR reaction).

To determine whether HGF and/or Met were expressed in neurons or in nonneuronal cells, we performed *in situ* hybridization on serial sections through the adult mouse SCG using digoxigenin-labelled riboprobes. Met (Fig. 2A) and HGF (Fig. 2B) receptor mRNAs were expressed in most, if not all, sympathetic neurons of the SCG. Moreover, both HGF and Met mRNAs were clearly localized to the same neurons (for example, see neuron denoted by

arrows in Figs. 2A and 2B; bar for Figs. 2A-2C is on Fig. 2C, and equals 75 μm for Figs. 2A-2B and 36 μm for Fig. 2C.). The specificity of this analysis was determined by hybridizing adjacent sections with sense Met or HGF riboprobes, neither of which produced any detectable signal.

To confirm that Met protein was also expressed in the mouse SCG, lysates of the postnatal day 1 (Fig. 1C) or adult SCG were precipitated either with a previously-characterized antibody to Met (lanes marked Liver + pep, Liver, and Sym neurons), or with wheat germ agglutinin (lane marked P1 SCG + WGA), which binds glycosylated proteins. These precipitates were then separated on polyacrylamide gels (SDS PAGE), transferred to nitrocellulose, and the filters incubated with the same Met antibody. This analysis revealed a Met-immunoreactive band of 145 kD in both the SCG and in the adult liver (Fig. 1C; size markers are shown to the left). This immunoreactive band was abolished when the Met antibody was first incubated with the Met immunizing peptide (Fig. 1C; Liver + pep), demonstrating its specificity. Immunocytochemistry confirmed that, like Met mRNA, Met protein was localized to sympathetic neurons of the SCG (Fig. 2C, dark reaction product); Met-like immunoreactivity was distributed throughout the ganglion, both in fibers coursing through the body of the ganglion (Fig. 2C, small arrowhead), as well as in sympathetic neuron cell bodies (Fig. 2C, large arrowhead; bar = 36 μm). No staining was observed when the antibody was preabsorbed with the immunizing Met peptide prior to immunocytochemistry. Thus, mature sympathetic neurons coexpress HGF and its receptor, the Met tyrosine kinase, in vivo.

To determine the role of the coexpressed HGF and Met, we turned to cultures of pure (>95%) neonatal rat sympathetic neurons from the SCG. We first used RT-PCR to confirm that cultured sympathetic neurons also expressed

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HGF and Met receptor mRNAs (Figs. 1A and 1B). We next determined whether neonatal sympathetic neurons expressed the Met receptor protein, as predicted by their synthesis of Met receptor mRNA. Cellular lysates of cultured neonatal sympathetic neurons were immunoprecipitated with anti-Met and the precipitates were analyzed by Western blot analysis with the same Met antibody. This analysis demonstrated that, like the intact SCG (Fig. 1C), cultured sympathetic neurons expressed a Met-immunoreactive band of the same size as that observed in liver (Fig. 1C).

To determine the spatial localization of this Met protein, we also performed fluorescent immunocytochemistry of cultured neonatal sympathetic neurons wing an antibody specific for Met (Fig. 2D; size bar for Fig. 2D-2F is on Fig. 2F, and equals 30 μm for Fig. 2D and 157 μm for Figs. 2E and 2F.); this analysis demonstrated that virtually all of the cultured neurons expressed Met, and that the Met-immunoreactivity was localized to both neurites and cell bodies (the arrow indicates a cell body). This immunostaining was abolished when the Met antibody was first preabsorbed with the immunizing Met peptide.

Finally, we determined if bioactive HGF was synthesized and secreted by sympathetic neurons using scatter assays, which take advantage of the fact that HGF causes cultured MDCK cells to become motile and "scatter" (Stoker et al., *supra*). Sympathetic neurons were cultured in NGF for 4 days, washed thoroughly, and then switched into new medium containing 10 ng/ml NGF. Twenty-four hours later, the sympathetic neuron conditioned media was removed, diluted at 1:100 into DMEM media, and transferred onto MDCK cells, which grow as tight clusters under standard culture conditions (Fig. 2E shows a phase-contrast photomicrograph of MDCK cells in control medium containing 10 ng/ml NGF). Twenty-four hours after MDCK cells were transferred to sympathetic neuron-conditioned media, they scattered in a

manner similar to that obtained with exogenous HGF (Fig. 2F shows a phase-contrast photomicrograph of MDCK cells in neuron-conditioned medium containing 10 ng/ml NGF; arrow shows scattering) (Stoker et al., *supra*). In contrast, no scattering was observed when unconditioned media, with or without 10 ng/ml NGF, was added to MDCK cells. Thus, cultured sympathetic neurons synthesize and secrete bioactive HGF-like scattering activity, most likely HGF itself.

Example III: Exogenous HGF Stimulates Immediate Early Gene Expression, but Not Survival of Sympathetic Neurons

The coexpression of HGF and the Met receptor in sympathetic neurons raised the possibility that HGF may function as an autocrine neurotrophic factor for these neurons. As a first step in investigating this possibility, we determined whether HGF was able to stimulate a functional Met receptor-mediated signaling response, as monitored by the immediate early gene c-fos. Previous work has demonstrated that HGF leads to an immediate activation of c-fos expression in epithelial cells and in septal neurons (Fabregat et al., *Biochem. Biophys. Res. Commun.* 189:684-690, 1992; Jung et al., *J. Cell Biol.* 126:485-94, 1994). To perform these experiments, sympathetic neurons of the postnatal day 1 SCG were acutely dissociated (Wyatt and Davies, *J. Cell Biol.* 130:1435-46, 1995), exposed for 3 hours to: no added neurotrophin (Fig. 3A), 10 ng/ml NGF (Fig. 3B), 10 ng/ml recombinant human HGF (rhHGF), or rhHGF pre-absorbed with an anti-HGF antibody (Genentech), and analyzed immunocytochemically for c-fos expression (Fig. 3A-3D; bar = 45 μm). This analysis demonstrated that HGF was capable of eliciting a robust induction of c-fos in approximately 80-90% of the sympathetic neurons in these acutely dissociated cultures (Fig. 3D), a response equivalent to that invoked by 10

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ng/ml NGF (Fig. 3A). These data, together with the immunocytochemical data, indicate that the vast majority of cultured sympathetic neurons express the Met receptor and can respond biologically to HGF.

We next determined whether Met signaling induced by HGF binding could support the survival of NGF-dependent sympathetic neurons. Neurons were selected for 5 days in 50 ng/ml NGF, washed thoroughly with neurotrophin-free medium, switched to media containing various concentrations of NGF or rhHGF, and survival measured 2 days later using MTT assays (Belliveau et al., *supra*). Figs. 4A-4C show the results of colorimetric MTT assays to measure mitochondrial function and cell survival. In the experiment represented in Fig. 4A, neonatal sympathetic neurons were cultured in 50 ng/ml NGF for 5 days, washed free of neurotrophin-containing medium, and then switched for 2 days to various concentrations of NGF or rhHGF as indicated on the X axis. Each point represents the values pooled from at least 3 separate survival assays, each of which was performed in triplicate. In these assays, absolute values are normalized so that the value obtained with 0 neurotrophin is 0% survival, while that obtained with 10 ng/ml NGF is considered 100% survival. Error bars represent SEM, and (**) denotes those values that were significantly different from the survival mediated by no added growth factors for two days, whereas ** denotes values significantly different from 2.5 ng/ml NGF ($P<0.001$).

In the experiments represented in Figs. 4B and 4C, neonatal sympathetic neurons were cultured as described for Fig. 4A after which they were switched to 1 ng/ml NGF plus or minus function-blocking anti-HGF obtained from Sigma (Fig. 4B) or from Genentech (Fig. 4C), as indicated on the X axis. Asterisks denotes those values significantly different from the survival mediated by 1 ng/ml NGF alone (**$P<0.001$). Neither antibody

significantly affected survival. Nonimmune serum (GS), at a concentration similar to that used for the experiments with the HGF antiserum (Genentech; Fig. 4C), also did not significantly affect sympathetic neuron survival. The MTT analyses indicated that concentrations of HGF of up to 100 ng/ml were unable to support survival of NGF-dependent sympathetic neurons (Fig. 4A). Similarly, when 100 or 200 ng/ml of rhHGF were added to sympathetic neurons immediately upon plating, no neurons survived, indicating the absence of an HGF-dependent population of neurons in the SCG.

Example IV: Exogenous HGF Selectively Promotes Sympathetic Neuron Growth

To test the possibility that HGF might be promoting neuronal growth rather than survival, we examined neurite extension in cultures of sympathetic neurons maintained in 10 ng/ml NGF. Figs. 4D-4F show quantitative analyses of neuritic process density in sympathetic neuron cultures grown in the presence of: NGF alone, NGH + HGF (Fig. 4D), KC1 + HGF (Fig. 4E), or NGF + anti-HGF antibody (Fig. 4F).

Specifically, sympathetic neurons were plated at low density for 1 day (Fig. 4D, experiment 1-3) or 4 days (Fig. 4D, experiment 4) in the presence of 10 ng/ml NGF. This concentration of NGF mediates 100% sympathetic neuron survival, but elicits limited morphological growth and TrkA activation relative to higher concentrations of NGF (Ma et al., *supra*; Belliveau et al., *supra*). Cultures were then switched to fresh media containing 10 ng/ml NGF with or without 30 ng/ml rhHGF (Fig. 4D experiments 1-3), 10 unit/ml of purified hHGF (Fig. 4D experiment 4) or, for comparison, 30 ng/ml NGF (a total of 40 ng/ml NGF; Fig. 4D). Two days later, fields were randomly selected, and the process network density was determined. In four separate

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experiments, the process network density was increased 2.0-fold ± 0.10 (**p<0.001) in the presence of 10 unit/ml or 30 ng/ml of HGF versus NGF alone (Fig. 4D, right panel; Figs. 5A-5B). Similarly, 40 ng/ml NGF increased neurite density approximately 2.3-fold relative to 10 ng/ml NGF alone (Fig. 4D, right panel), an increase similar to the 2 to 2.5-fold increase we have previously documented for 30 ng/ml NGF relative to 10 ng/ml NGF (Belliveau et al., *supra*). Thus, when neuronal survival is maintained with NGF, exogenous HGF enhances sympathetic neuron growth to approximately the same degree as addition of a similar amount of exogenous NGF (Fig. 4D, right panel), the most potent growth factor known for these neurons.

These experiments indicate that exogenous HGF promotes sympathetic neuron growth in the presence of NGF, which itself promotes neuronal growth. To determine whether HGF could promote sympathetic neuron growth on its own, we performed similar assays using KCl, an agent that maintains sympathetic neuron survival without promoting growth (Franklin et al., *J. Neurosci.* 15:643-664, 1995). Specifically, neurons were grown for 4 days in 10 ng/ml NGF, and then, after extensive washing, were switched to 50 mM KCl with or without 30 or 100 ng/ml HGF, or 10 ng/ml NGF (Fig. 4E; Fig. 5D-5F). Two days later, fields were randomly selected, and the process network density was determined. In four separate experiments, the process network density was increased approximately 1.5-fold ± 0.09 and 2.6-fold ± 0.16 (**p<0.001) for 30 and 100 ng/ml HGF, respectively (Fig. 4E, right panel; Fig. 5D-5E). By comparison, addition of 10 ng/ml NGF increased neurite density by 3.2-fold ± 0.19 (Fig. 4E, right panel; Fig. 5D, 5F). Thus, HGF not only enhances sympathetic neuron growth in the presence of NGF, but can also promote neurite extension on its own.

The left panel of Fig. 4F shows the results of (Fig. 4F, left panel)

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three separate experiments that were performed to determine the effect of blocking endogenous HGF on process density in sympathetic neurons. Sympathetic neurons were plated at low density on collagen for 1 day in 10 ng/ml NGF, and then were switched for 2 days to 10 ng/ml NGF with or without 5 μg/ml of Sigma anti-HGF or 5 μl/ml Genentech HGF antiserum (anti-HGF). As a control, neurons were also switched to NGF containing 5 μl/ml nonimmune goat serum (GS). In all three experiments, significantly fewer neurite intersections were observed when endogenous HGF was neutralized with an antibody to HGF (**P<0.001). In the right panel of Fig. 4F, the data shown for each experiment in (the left panel of Fig. 4F) have been normalized so that the neuritic density at 10 ng/ml NGF is 1.0, and then combined to provide an index of the relative neurite density following each treatment (**P<0.001 relative to 10 ng/ml NGF alone).

Fig. 5A-5F are phase contrast micrographs showing that endogenous HGF is necessary for optimal growth of cultured sympathetic neurons. Figs. 5A-5C show neurons maintained in 10 ng/ml NGF for 1 day and then switched to (Fig. 5A) 10 ng/ml NGF, (Fig. 5B) 10 ng/ml NGF plus 30 ng/ml rhHGF, or (Fig. 5C) 10 ng/ml NGF plus 5 μl/ml HGF antiserum (Genentech). Exogenous HGF enhanced and HGF antibody decreased process outgrowth. Fig. 5D-5F show neurons maintained in 10 ng/ml NGF for 4 days, and then switched to (Fig. 5D) 50 mM KCl, (Fig. 5E) 50 mM KCl plus 100 ng/ml HGF, or (Fig. 5F) 50 mM KCl plus 10 ng/ml NGF. The magnification bar equals 100 μm in all panels.

We next determined whether HGF could promote the rate of forward axonal extension, a second index of sympathetic neuron growth. To measure this parameter, we turned to compartmented cultures of sympathetic neurons, a system (Campenot, *Dev. Biol.* 93:1-12, 1982a; Campenot, *Dev. Biol.* 93:13-21,

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1982b; Campenot, "Compartmented culture analysis of nerve growth." In: *Cell-Cell Interactions: A Practical Approach*. Stevenson et al. Eds. Oxford Univ. Press, Oxford pp 275-298, 1992) that allows i) measurement of the rate of forward axonal growth, and ii) independent manipulation of the environment of distal neurites versus that of proximal neurites and cell bodies, thereby allowing for an analysis of local effects on axonal growth. To perform these experiments, we established compartmented cultures with 10 ng/ml NGF in the center compartment, 1 ng/ml NGF in one side compartment, and 1 ng/ml NGF plus 30 ng/ml rhHGF in the other side compartment. The length of axons in the side compartments was then measured every second day for six days (Fig. 6A). Plots represent the combined results from three sister cultures showing the average length of neurites at 2.5, 4.5, and 6.5 days following establishment. Error bars indicate standard error of the mean, and asterisks denote those timepoints where growth was significantly different between the experimental and control sides (**$P<0.001$). This analysis revealed that HGF applied to neurites was capable of modestly enhancing the forward rate of neurite outgrowth over the entire six day period (Fig. 6A); by the sixth day, there was an increase of approximately 18% in the average length of neurites that were exposed to NGF plus HGF versus NGF alone.

In a second set of compartmented culture experiments, we measured the effects of exogenous HGF on the forward rate of axonal extension in the presence of KCl. Specifically, cultures were established with 10 ng/ml NGF in all compartments for 4 days, and then were switched to 50 mM KCl with or without 30 ng/ml HGF in the side compartments. The extent of forward axonal growth was measured immediately following the switch, and then two days later (Fig. 6B). The bar graphs represent the average total length of axon extension (error bars represent S.E.) over two days in KCl with or without

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HGF. A similar HGF-mediated increase was observed in two separate experiments (**P<0.001; n = 3 cultures for each treatment in each experiment).

Figs. 6C and 6D show that endogenous local HGF is necessary for optimal axonal extension rate. In the experiment shown in Fig. 6C, compartmented cultures were established with 10 ng/ml NGF in all compartments, and 5 µl/ml HGF antiserum in one side compartment. In Experiment 1, the results from measurements of neurite length in three sister cultures at 3.5, 4.5, and 5.5 days with (anti-HGF) or without (Control) anti-HGF were combined. In Experiment 2, the results from measurements of neurite length in the side compartments of three sister cultures at 4, 6, and 7 days were combined. In both experiments, error bars and significance are as in Fig. 6A. In some cases, the error bars fall within the symbols. In the experiment shown in Fig. 6D, compartmented cultures were initially established with 10 ng/ml NGF in the center compartment and 3 ng/ml NGF in both side compartments. At 2 days post-plating, a timepoint when all neurites had crossed, 3 ng/ml NGF plus 5 µl/ml anti-HGF antiserum (Genentech) was added to one side compartment and 3 ng/ml plus 5 µl/ml nonimmune sheep serum to the other, and neurite lengths were measured immediately. Neurite length was again measured on days 2,4,5, and 6; at 3 days, media was replaced with new media containing the same concentrations of NGF, anti-HGF and nonimmune serum. Results represent data combined from 6 different cultures from two separate experiments. Errors and significance are as in Fig. 6A.

In the experiment shown in Fig. 6E, anti-HGF in the central compartment does not affect the amount of forward axonal growth in the side compartments. Cultures were established with 10 ng/ml NGF in all compartments with or without the addition of 5 µl/ml anti-HGF in the central compartment. Plots represent the results obtained in two separate experiments

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in which sister cultures (three each with and without anti-HGF) were measured at 2, 5, and 6 days in Experiment 1 and 4, 6, and 7 days in Experiment 2. Error bars and significance are as in Fig. 6A. The amount of axonal extension was not significantly affected by anti-HGF in either experiment ($P>0.05$).

To control for the effects of rat serum, cultures were established with 10 ng/ml NGF plus 3% rat serum in all compartments with 5 µl/ml anti-HGF in one side compartment. The plot shown in Fig. 6F represents the combined data from measurements of neurite length in the compartments with and without anti-HGF in three sister cultures at 4, 6, and 7 days. Error bars and significance are as in Fig. 6A. Note that this experiment was performed on sister cultures to those shown in panel C, Experiment 2, demonstrating that anti-HGF had similar effects whether the side compartments contained serum (Fig. 6F) or not (Fig. 6C, Experiment 2).

To determine whether the anti-HGF effect was dependent upon the substrate, cultures were established on a poly-D-lysine/laminin substratum with 10 ng/ml NGF in all compartments and 5 µl/ml anti-HGF in one side compartment. In Experiment 1 of Fig. 6G, the plot represents the combined measurements of neurite length from three sister cultures at 4, 5, and 6 days in side compartments with or without anti-HGF. In Experiment 2, the plot represents the combined data from four sister cultures that were measured at 5 days. Error bars and significance are as in Fig. 6A.

The analyses described above demonstrated that HGF was capable of promoting forward axonal growth in the absence of NGF, although this effect (Fig. 6B) was not as robust as the effect on neurite density (Fig. 4E). Together these data indicate that HGF can promote increased neuritic density and forward axonal extension in the presence or absence of NGF. Moreover, our compartmented culture data indicate that HGF can act locally through axonal

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Met receptors to enhance axonal growth.

Example V: Autocrine HGF is Essential for Optimal Morphological Growth, but not Survival of Sympathetic Neurons

Together, these data demonstrate that activation of the Met receptor with exogenous HGF can promote neurite growth independent of an effect on neuronal survival. To determine whether autocrine HGF played a similar role, we inhibited endogenous sympathetic neuron-derived HGF using two different function-blocking HGF antibodies (one commercially-available purified anti-HGF IgG from Sigma (Rubin et al., *Proc. Natl. Acad. Sci. USA* 88:415-419, 1991), and one an anti-HGF antiserum that was the kind gift of Genentech (Tsao et al., *Cell Growth Differ.* 4:571-579, 1993). Initially, we confirmed the previously-reported ability of these antibodies to neutralize HGF by determining whether they inhibited the induction of c-fos by exogenous HGF. Specifically, 10 ng/ml HGF was preincubated with the anti-HGF at 4° C for three hours, was added to acutely-dissociated sympathetic neurons, and c-fos induction was monitored by immunostaining (Fig. 3C). As a control, we used 10 ng/ml HGF that was not preabsorbed (Fig. 3D). This analysis confirmed that both function-blocking HGF antibodies inhibited the ability of exogenous HGF to induce neuronal c-fos expression.

We then used these antibodies to determine whether endogenous HGF played any role in sympathetic neuron survival or growth. For the survival experiments, sympathetic neurons were cultured for 5 days in 50 ng/ml of NGF, and were then switched into suboptimal concentrations of NGF with or without anti-HGF (5 μg/ml for Sigma anti-HGF and 5 μl/ml for Genentech anti-HGF). Addition of either the anti-HGF IgG (Sigma) (Fig. 4B) or the anti-HGF antiserum (Genentech) (Fig. 4C) had no effect on sympathetic neuron

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survival as mediated by 1 ng/ml (Fig. 4B-4C) or 5 ng/ml NGF. Thus, endogenous HGF is not apparently required for NGF-mediated sympathetic neuron survival.

To determine whether endogenous HGF was necessary for neuronal growth, we assayed both neurite density and forward axonal growth rate. To examine effects on density, mass cultures of sympathetic neurons were cultured for one day in defined media containing 10 ng/ml NGF, and then were switched into NGF-containing media with or without anti-HGF. Two days later, these cultures were analyzed for neurite process density (Fig. 4F; Fig. 5A-5C). This analysis demonstrated that both of the function-blocking HGF antibodies decreased neurite density by 2.5 to 3-fold (Fig. 4F, Fig. 5C) relative to 10 ng/ml NGF alone (Fig. 4F, Fig. 5A), or relative to 10 ng/ml NGF plus nonimmune serum (Fig. 4F). The magnitude of this decrease was similar to that observed in neurons maintained in 50 mM KCl alone (which does not support growth) relative to those in 50 mM KCl plus 10 ng/ml NGF (Fig. 4E). Thus, the addition of anti-HGF reduced NGF-promoted neurite density to approximately the same degree as switching these neurons from NGF into a survival factor that does not promote growth.

To determine whether autocrine HGF was also necessary for the NGF-promoted rate of forward axonal growth, we performed similar experiments in compartmented cultures. Specifically, compartmented cultures were established with 10 ng/ml NGF in the center compartment and one of the side compartments, and 10 ng/ml NGF plus 5 µl/ml anti-HGF (Genentech) in the other side compartment. The amount of axonal growth was then measured at 3.5, 4.5 and 5.5 days (Experiment 1, Fig. 6C) or at 4, 6, and 7 days (Experiment 2, Fig. 6C). In both of these experiments, the rate of forward axonal growth was significantly decreased in the compartment containing the

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HGF antibody relative to axons of the same neurons extending into the control side compartment (Fig. 6C). At days 5.5 (Experiment 1) and 7 (Experiment 2), the total extension length was decreased an average of 30% and 34%, respectively, when endogenous HGF was neutralized. As an additional control, we performed experiments where compartmented cultures were established as above, with one side containing 3 ng/ml NGF plus 5 μl/ml nonimmune sheep serum and the other containing 3 ng/ml NGF plus 5 μl/ml anti-HGF. In these experiments (Fig. 6D), at 4 days following addition of anti-HGF, the total extension length was decreased an average of 20% in the side containing anti-HGF relative to that containing nonimmune serum. Together, these experiments indicate that autocrine HGF is necessary for optimal expression of two different facets of neuronal growth, neuritic density and the forward rate of axonal growth.

Example VI: Autocrine HGF Promotes Axonal Extension in a Local, Substrate-Independent Fashion

The compartmented culture results indicated that axonally-produced HGF acted locally to promote an optimal axonal extension rate. However, endogenous HGF could also be promoting sympathetic neuron growth by acting globally, for example, to increase the expression of genes important for neuronal growth (Ma et al.,*supra*; Belliveau et al., *supra*). To test this possibility, we neutralized autocrine HGF in the center compartment of compartmented cultures, which contain neuronal cell bodies and proximal neurites, and determined whether this affected the rate of axonal extension in the side compartments, which contain distal axons. Specifically, compartmented cultures were established with 10 ng/ml NGF in all compartments, and then anti-HGF (Genentech) was added to the central

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compartments of half of the sister cultures. The amount of neurite extension was then measured at 4, 5, and 6 days (3 cultures each treatment, Experiment 1, Fig. 6E) or 4, 6, and 7 days (3 cultures each treatment, Experiment 2, Fig. 6E). These experiments indicated that the amount of axonal extension was not significantly altered by inhibiting endogenous HGF in the center compartment (Fig. 6E).

One difference between the center and side compartment environments is the presence of serum in the center compartment. To ensure that the lack of effect observed when anti-HGF was added to the center compartment was not due to this variable, we performed experiments where serum was added to the side compartments. Specifically, compartments were established with 10 ng/ml NGF and 3% serum in all compartments, and 5 µl/ml anti-HGF (Genentech) in one side compartment. The amount of neurite extension was measured at 4, 6, and 7 days (Fig. 6F). As observed without serum, the amount of forward axonal extension was significantly decreased in the presence of the function-blocking anti-HGF. At 4, 6, and 7 days, the amount of axonal growth was decreased an average of 67%, 53% and 48%, in the side compartment containing anti-HGF relative to the control side. Similar results were observed when 5 µl/ml nonspecific sheep serum was added to the side compartment that did not contain anti-HGF.

To determine whether this phenomena was substrate-dependent, we performed compartmented cultures studies on poly-D-lysine/laminin as opposed to collagen. As previously, compartments were established with 10 ng/ml NGF in all compartments, 5 µl/ml anti-HGF (Genentech) was added to one side compartment, and the amount of axonal growth was measured at 4, 5, and 6 days (Fig. 6G). These studies demonstrated that autocrine HGF promoted axonal growth through a substrate-independent mechanism; as

observed on collagen, neutralization of local endogenous HGF led to an average decrease in axonal growth of 22% throughout the entirety of the experiment (Fig. 6G, Experiment 1). This inhibition was confirmed in a second experiment where cultures were measured only at 5 days (Fig. 6G, Experiment 2; combined data of 4 cultures); in this case, axonal growth was decreased 18%.

Interestingly, in addition to the decrease in forward rate of growth, there was also a striking decrease in neurite density in the side compartment containing the anti-HGF relative to the control side. Figs. 7A-7B show phase contrast photomicrographs of neurites on a single track from a sympathetic neuron compartmented culture on poly-D-lysine/laminin where the left compartment (Fig. 7A) has been maintained in 10 ng/ml NGF, while the right compartment (Fig. 7B) has been maintained in 10 ng/ml NGF plus 5 µl/ml HGF antiserum (Genentech). The photographs were taken about 4 mm away from the silicone grease barrier that separates the central and side compartments. The scratches in the substratum that form the borders of the track are visible along the top and bottom portion of each panel. That the density of neurites in the side compartment containing anti-HGF is significantly lower. This difference was not obvious on a collagen substratum in compartmented cultures, possibly because the axons fasciculate to a greater degree. To obtain an idea of the total decrease in axonal growth caused by anti-HGF under these conditions, we isolated the total protein from the side compartments of these cultures and measured tubulin levels using Western blots. Side compartments from two of the cultures measured in Fig. 6G, Experiment 2 were collected on day 6, lysed, and analysed for total tubulin per side compartment. (Cult 1) refers to one culture and (Cult 2) to the second. One side compartment (Cult 1 or Cult 2) was treated with 10 ng/ml NGF alone

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for 6 days, while the other (Cult 1 + anti-HGF, Cult 2 + anti-HGF) was treated with 10 ng/ml NGF plus anti-HGF. The tubulin band is denoted as α-Tubulin.

The Western analysis shown in Fig. 7C revealed a dramatic decrease in the amount of total tubulin in the side compartments treated with anti-HGF versus those without, a decrease that was presumably due both to decreased neuritic density and to decreased forward extension. Thus, endogenous local HGF is essential for growth of sympathetic axons, promoting both the rate and density of axonal growth in a substrate-independent fashion.

Other Embodiments

The invention may be practiced with numerous variations including, but not limited to, those described below. The HGFs or HGF genes used in the methods of the invention may be naturally occurring, or may be produced using methods for recombinant DNA technology. HGFs or HGF genes may be derived from a variety of animal species, and may include naturally occurring or artificially mutated variants. Such sequence variants may have properties that are useful in the methods of the invention, including enhanced stability or biological activity. For example, sequence variants described in the U.S. patents "Single-Chain Hepatocyte Growth Factor Variants" (USPN 5,316,921) and "Hepatocyte Growth Factor Variants" (USPN 5,547,856) are resistant to proteolytic cleavage, and, therefore, are likely to be useful for increasing the effectiveness of HGF administered to prevent or treat axonal degeneration, such as that occurring in neuropathy.

Expression vectors such as adenoviral vectors may be used to express exogenous HGF genes in neurons or in non-neuronal cells. Such adenoviral vectors efficiently introduce DNA into cells such as post-mitotic neurons, as described in U.S. patent application "Post-Mitotic Neurons

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Containing Adenovirus Vectors That Modulate Apotosis and Growth" (USSN 60/066,761).

Expression of exogenously-introduced HGF genes may be limited to neurons by placing HGF gene expression under the regulation of a neuron-specific promoter. One such example is the promoter of the neuron-specific Tα1 α-tubulin gene, which is abundantly expressed in neurons during growth and target re-innervation. The Tα1 α-tubulin gene promoter is described in U.S. patent "Tα1 α-Tubulin Promoter and Expression Vectors" (USPN 5,661,032), and in U.S. patent application "Neuron Promoter and Uses" (USSN 08/900,026).

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth.

What is claimed is:

1. A method for promoting axonal growth or axonal regeneration of a post-natal neuron, said method comprising administering an expression vector to said neuron, said expression vector comprising a hepatocyte growth factor gene operably linked to a promoter.

2. A method for inhibiting axonal degeneration of a post-natal neuron, said method comprising administering an expression vector to said neuron, said expression vector comprising a hepatocyte growth factor gene operably linked to a promoter.

3. A method for treating or inhibiting neuropathy in a patient, said method comprising administering a therapeutically effective dose of hepatocyte growth factor to said patient.

4. A method for treating or inhibiting neuropathy in a patient, said method comprising administering an expression vector comprising a hepatocyte growth factor gene operably linked to a promoter to said patient.

5. The method of claim 3 or 4, wherein said patient is identified as having a neuropathy.

6. The method of claim 5, wherein said neuropathy is a symptomatic neuropathy.

7. The method of claim 3 or 4, wherein said neuropathy is an asymptomatic neuropathy.

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8. The method of claim 3 or 4, wherein said neuropathy is caused by axonal degeneration.

9. The method of claim 3 or 4, wherein said neuropathy is an autonomic neuropathy.

10. The method of claim 3 or 4, wherein said neuropathy is a sensory neuropathy.

11. The method of claim 3 or 4, wherein said neuropathy is a sensorimotor neuropathy.

12. The method of claim 3 or 4, wherein said neuropathy is a motor neuropathy.

13. The method of claim 4, wherein said expression vector is expressed in a neuron.

14. The method of claim 4, wherein said expression vector is expressed in a non-neuronal cell in the region of the body where said neuropathy is present.

15. The method of claim 1, 2, or 4, wherein said hepatocyte growth factor gene further encodes a signal sequence that directs secretion of said hepatocyte growth factor from said neuron or said non-neuronal cell.

16. The expression vector of claim 1, 2, or 4, said vector being an

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adenoviral vector.

17. The promoter of claim 1, 2, or 4, said promoter being a Tα1 α-tubulin promoter.

18. The method of claim 1, 2, 3, or 4, wherein said hepatocyte growth factor comprises a non-cleavable sequence variant.

19. The method of claim 1, 2, 3, or 4, wherein said HGF or said expression vector is administered to a neuron selected from: a sympathetic neuron, a parasympathetic neuron, a sensory neuron, or a motor neuron.

20. The method of claim 19, wherein said vector is administered to the terminals of sympathetic neurons.

21. The method of claim 3 or 4, wherein said patient is identified as at risk for diabetic neuropathy prior to said preventing.

22. The method of claim 3 or 4, wherein said patient is identified as having diabetic neuropathy.

23. The method of claim 22, wherein said patient is identified as having clinical manifestations of diabetic neuropathy prior to said treating.

24. The method of claim 21 or 22, wherein said diabetic neuropathy is caused by insulin-dependent diabetes.

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25. The method of claim 21 or 22, wherein said diabetic neuropathy is caused by non-insulin-dependent diabetes.

26. The method of claim 21 or 22, wherein said diabetic neuropathy is selected from: distal sensory polyneuropathy, sensorimotor polyneuropathy, autonomic neuropathy, visceral autonomic neuropathy, mononeuropathy, or mononeuropathy multiplex.

27. The method of claim 26, wherein said patient has a foot ulceration.

28. The method of claim 26, wherein said patient has a cardiac arrhythmia.

29. The method of claim 26, wherein said patient has sexual impotence.

30. The method of claim 26, wherein said patient has chronic pain.

31. The method of claim 26, wherein said diabetic neuropathy results in abnormal vascular responses.

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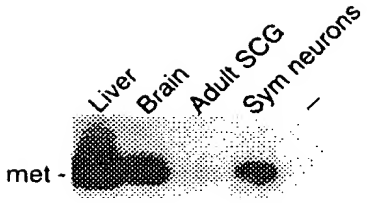

Fig. 1A

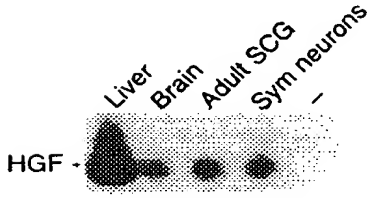

Fig. 1B

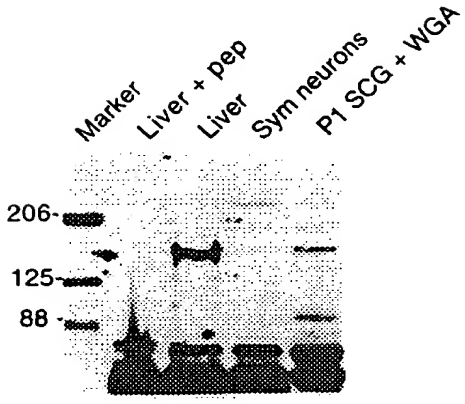

Fig. 1C

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Fig. 2A

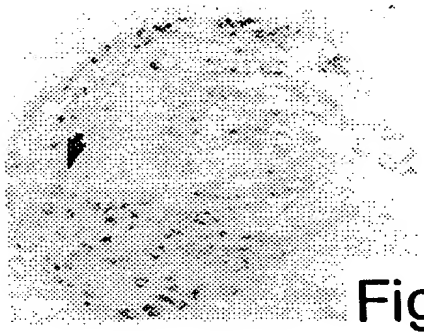

Fig. 2B

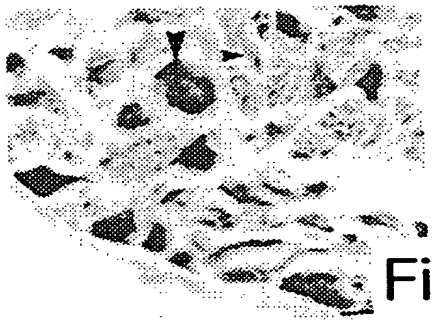

Fig. 2C

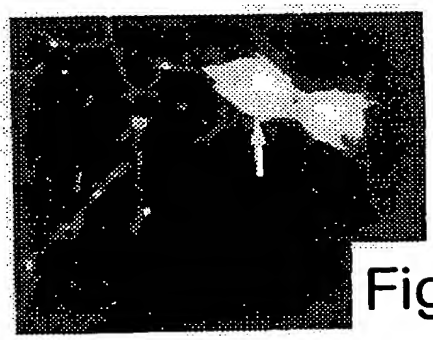

Fig. 2D

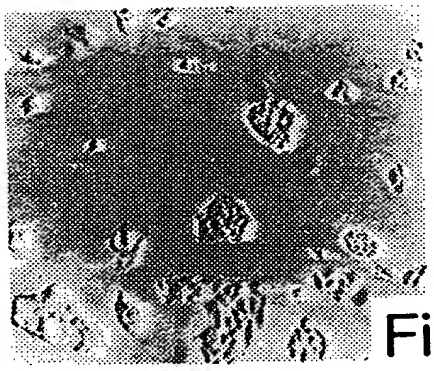

Fig. 2E

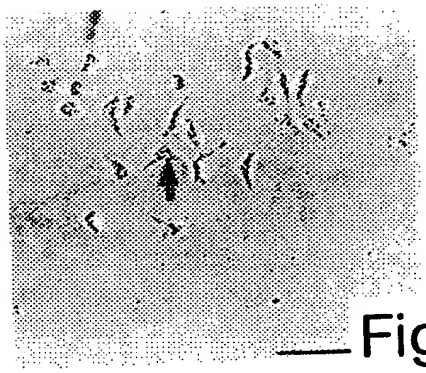

Fig. 2F

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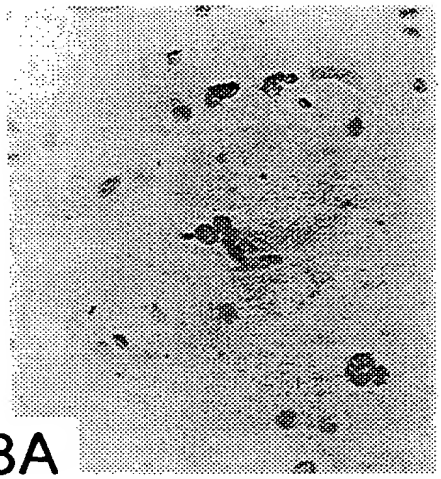

Fig. 3A

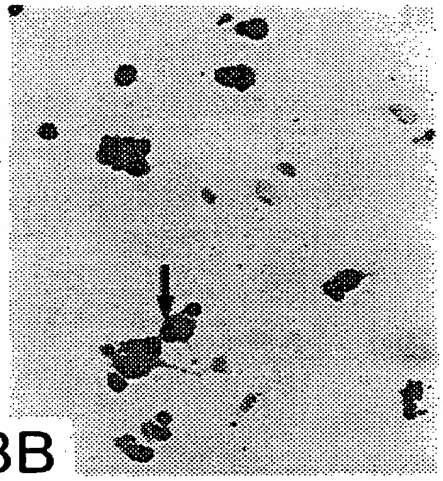

Fig. 3B

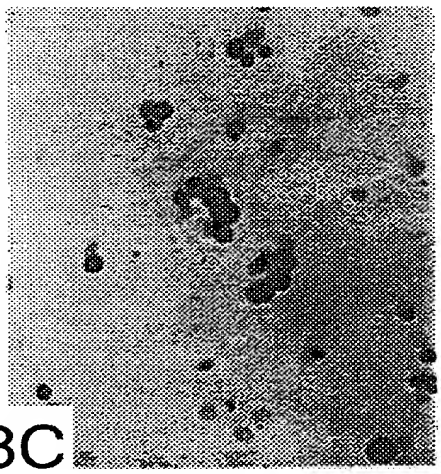

Fig. 3C

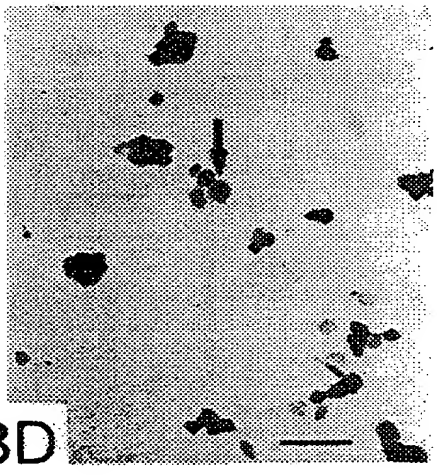

Fig. 3D

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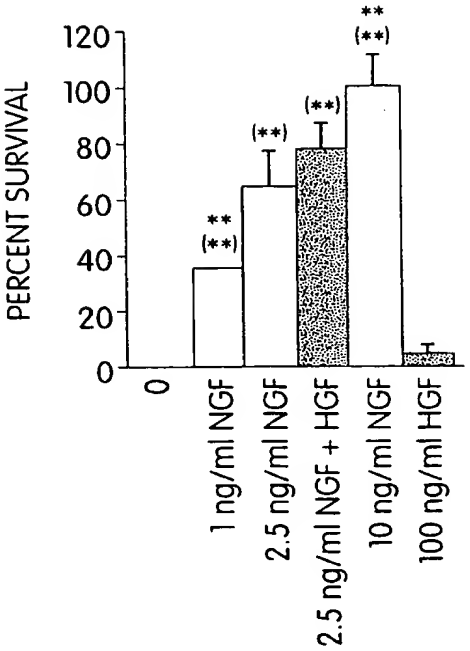

Fig. 4A

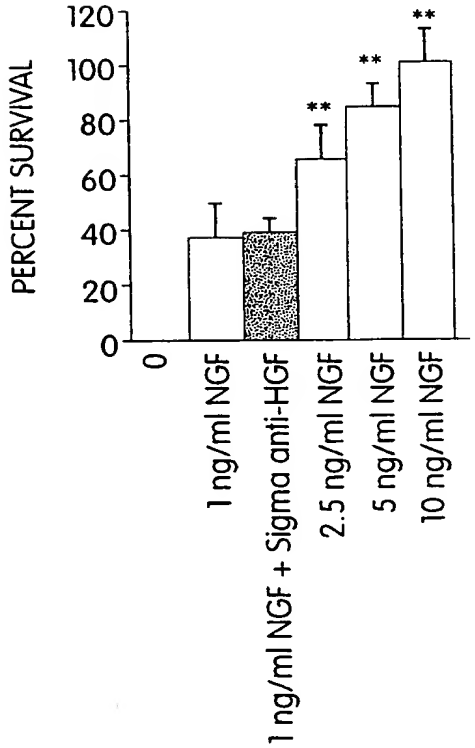

Fig. 4B

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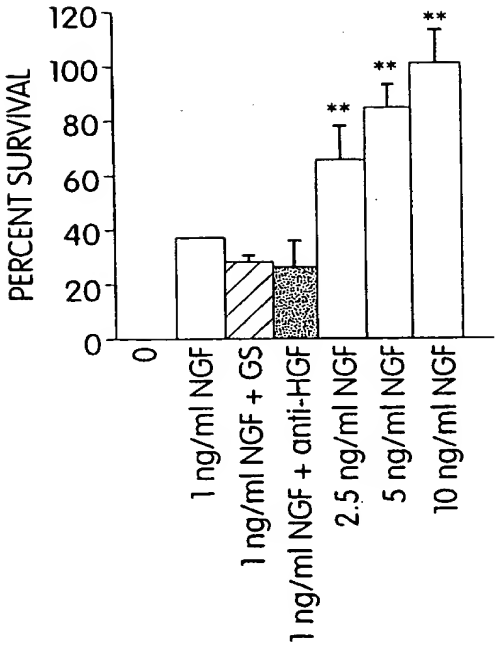

Fig. 4C

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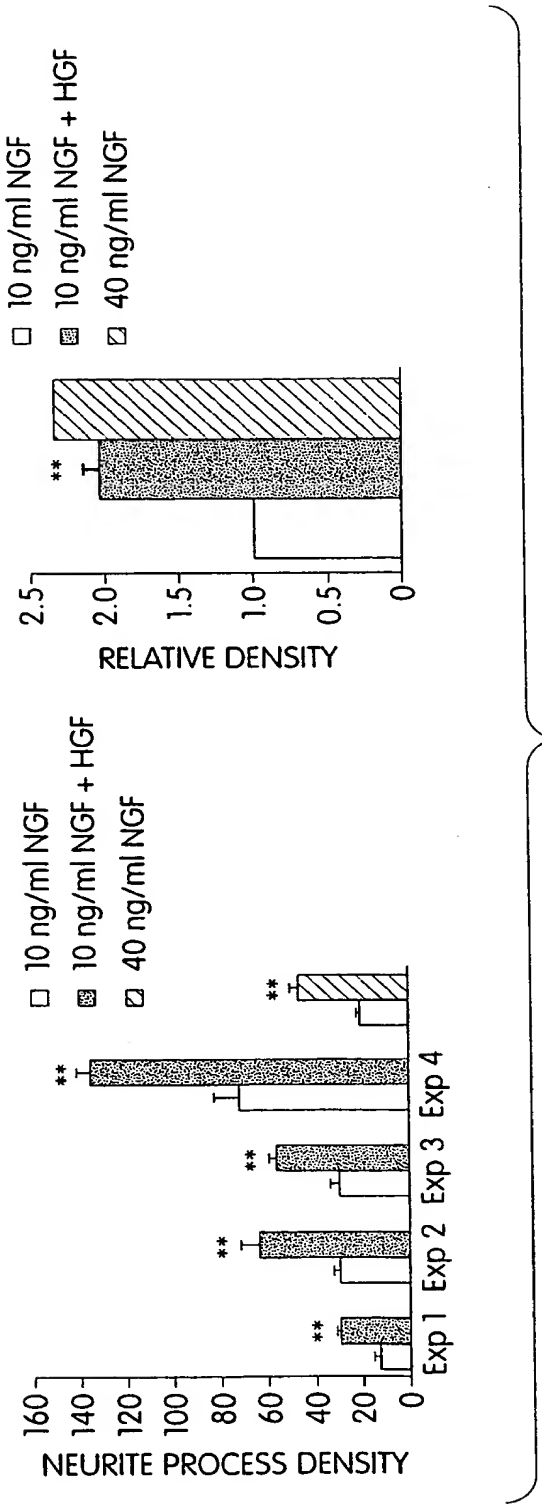

Fig. 4D

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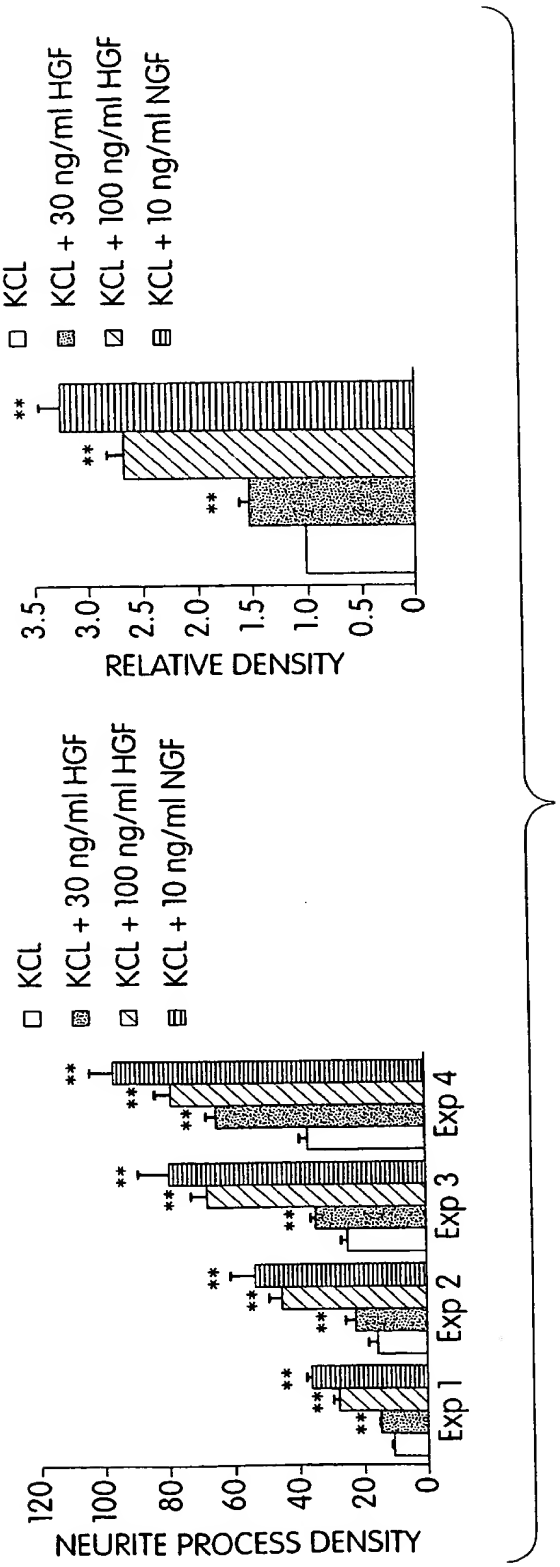

Fig. 4E

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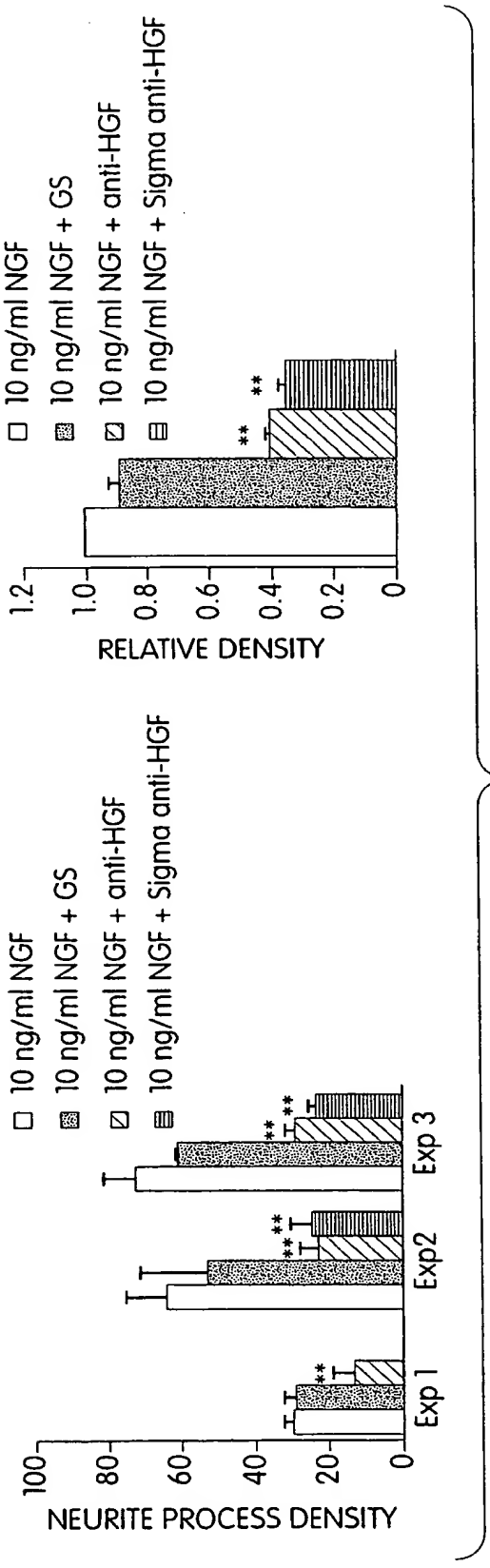

Fig. 4F

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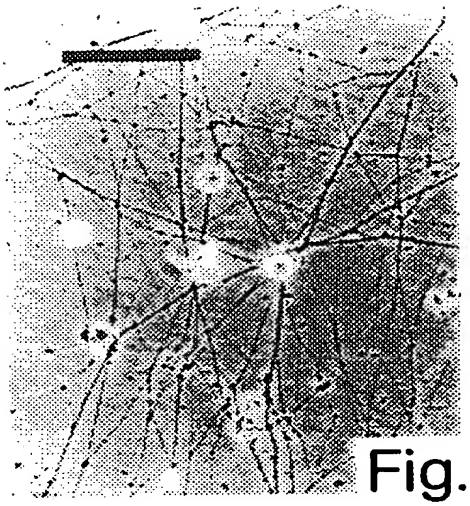


Fig. 5A

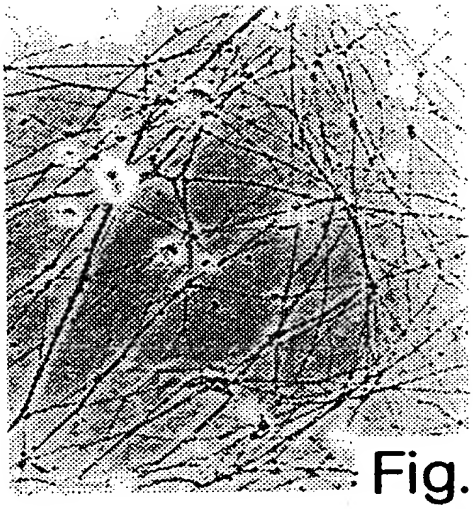


Fig. 5B

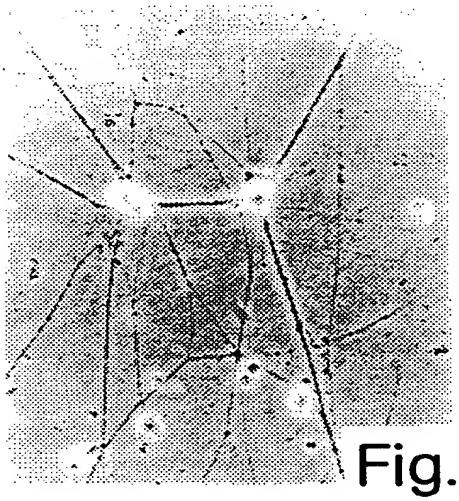


Fig. 5C

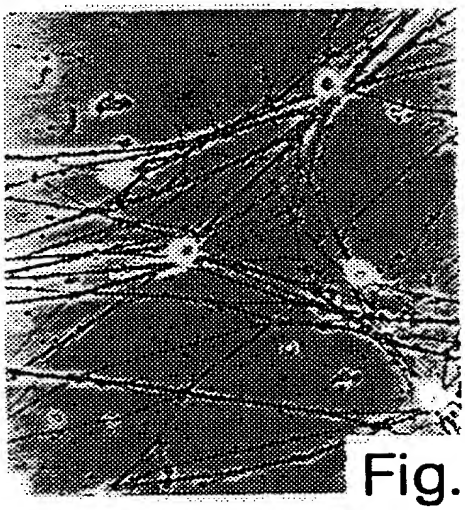
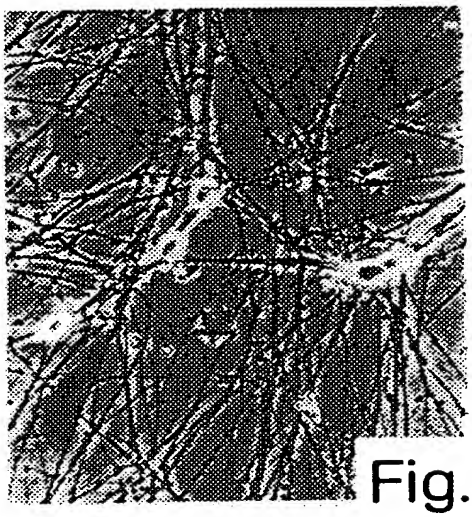


Fig. 5D



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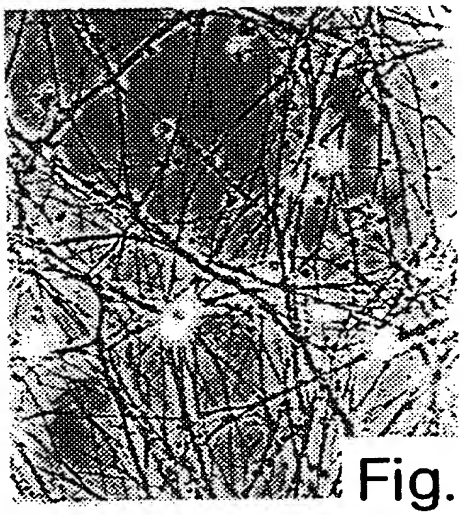

Fig. 5F

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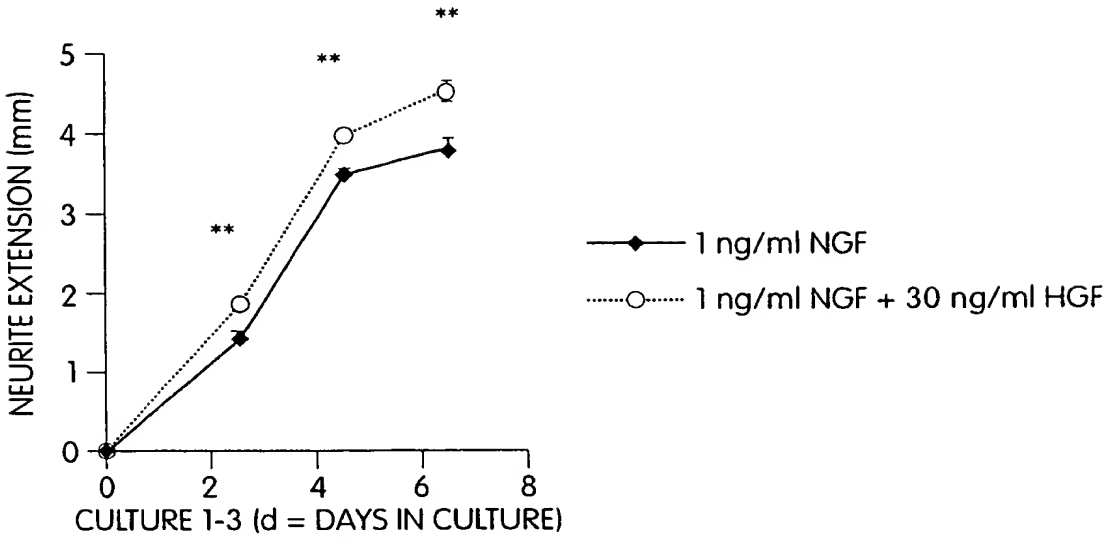

Fig. 6A

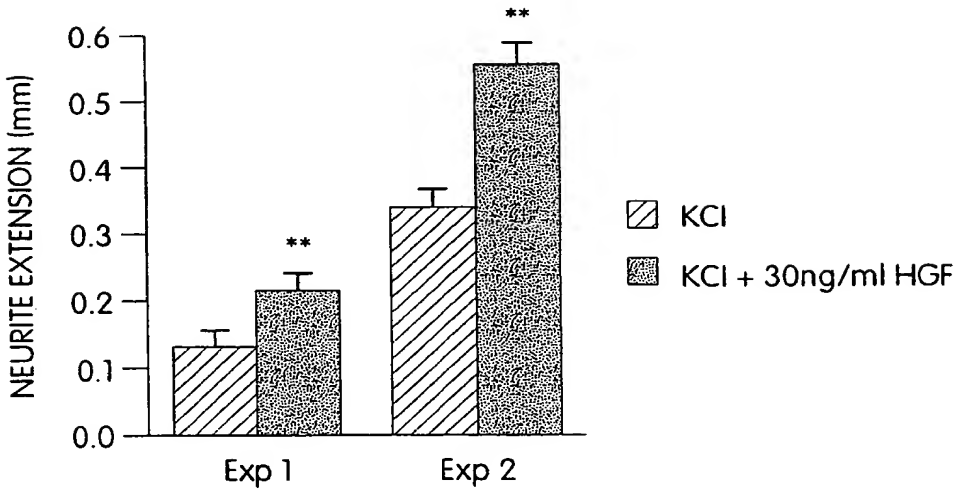

Fig. 6B

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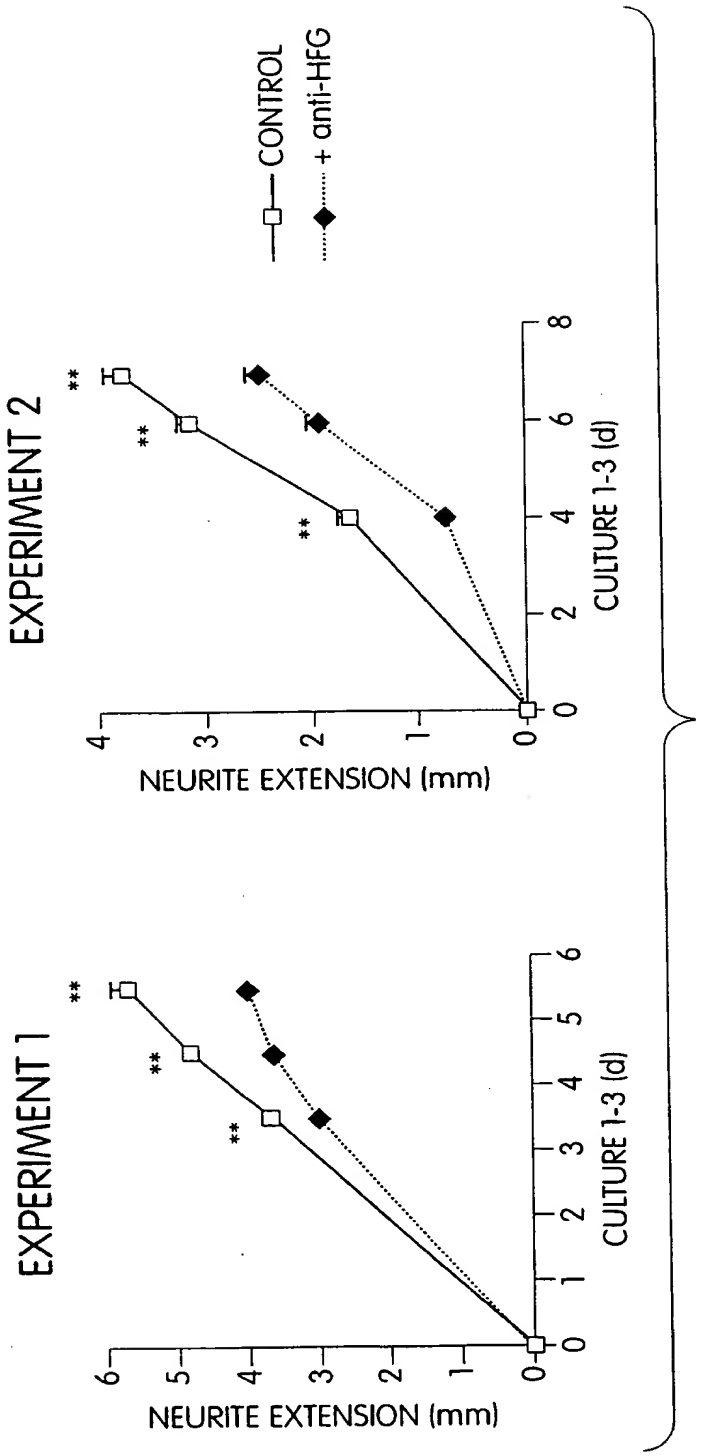

Fig. 6C

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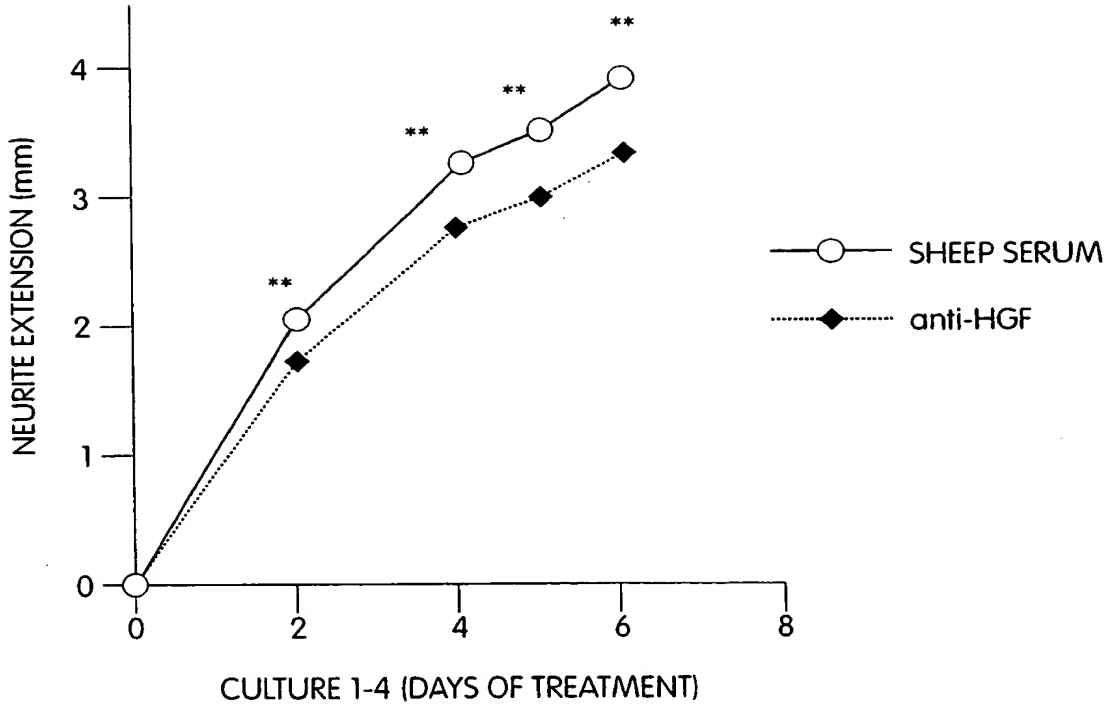

Fig. 6D

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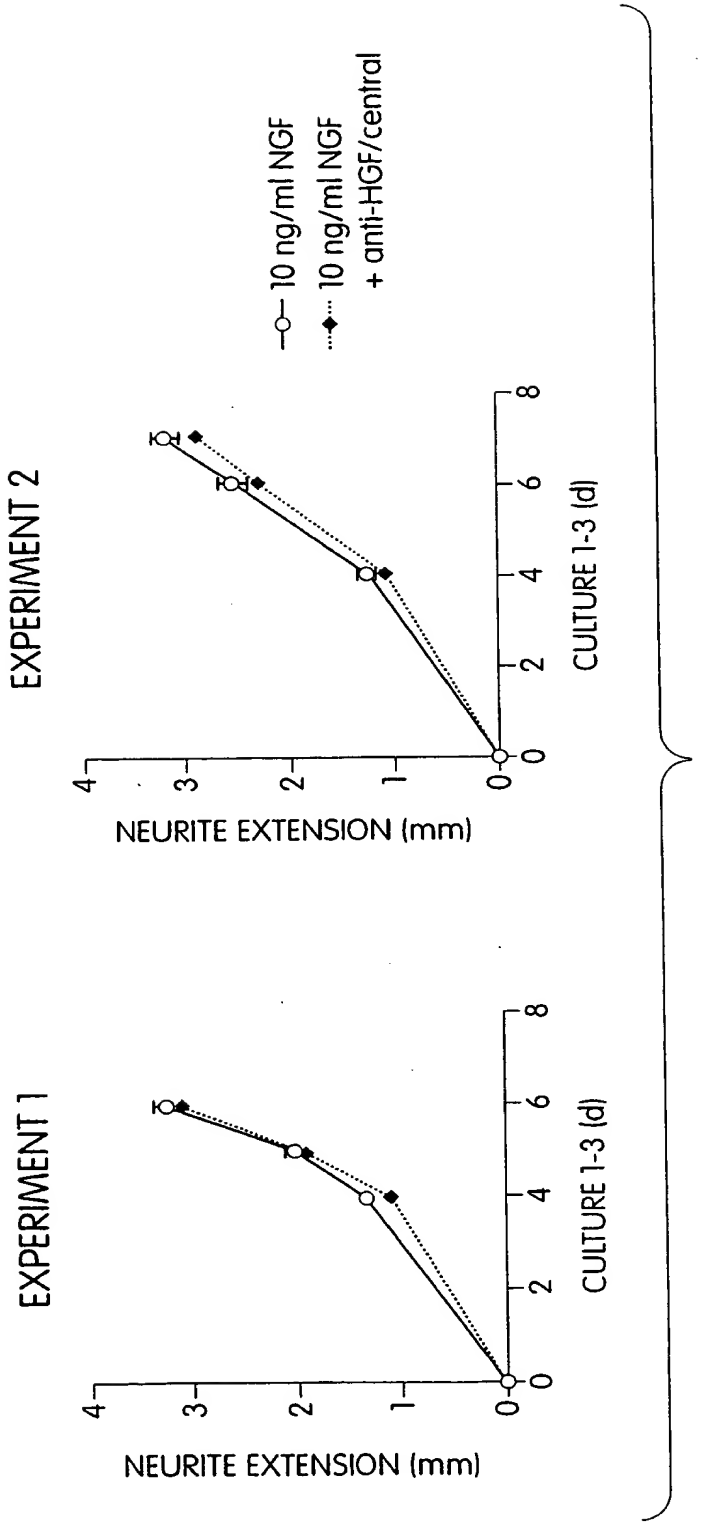

Fig. 6E

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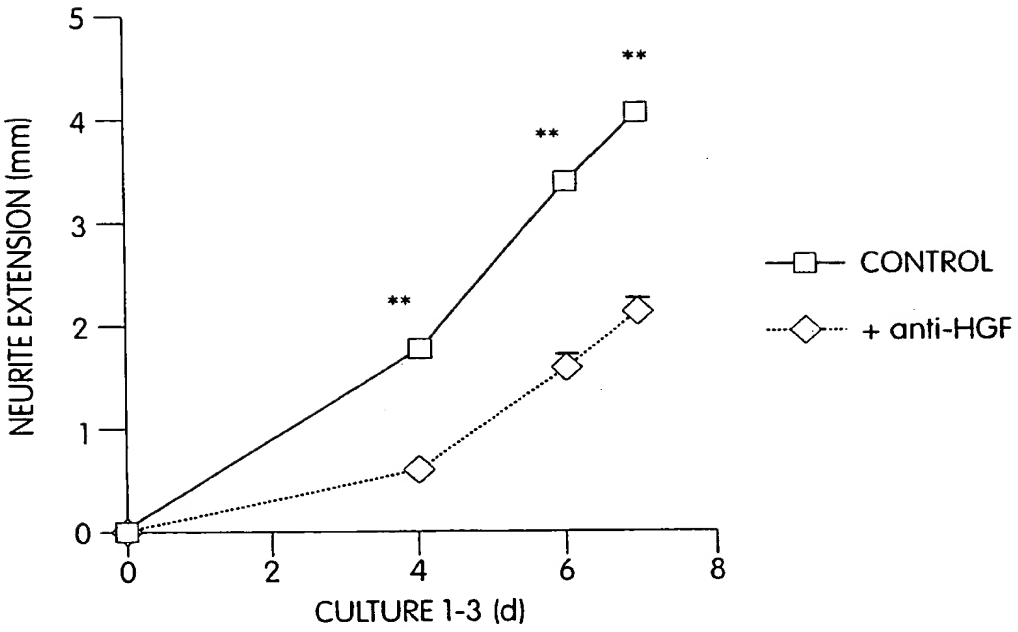

Fig. 6F

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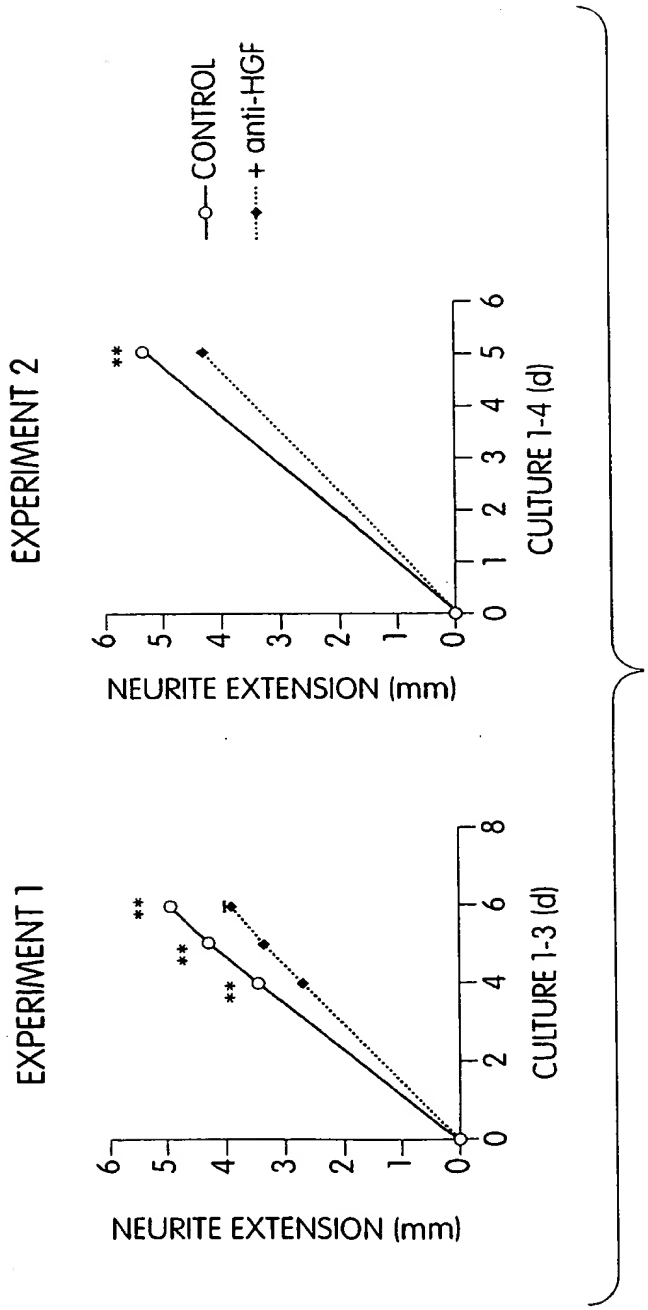

Fig. 6G

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Fig. 7A

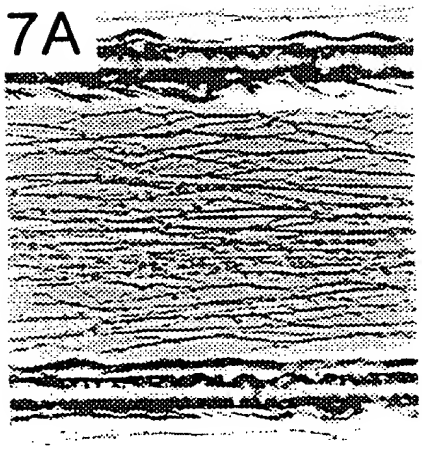


Fig. 7B

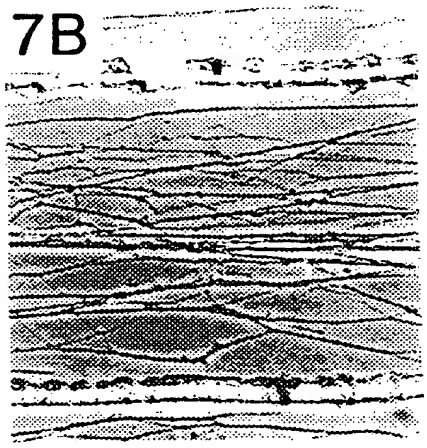
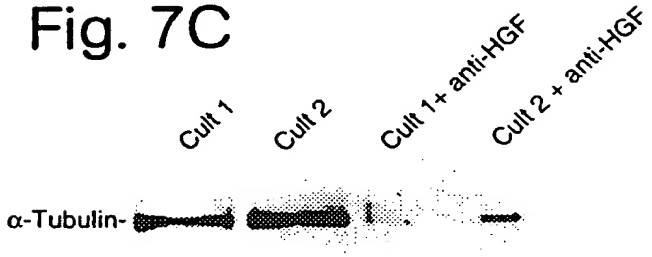


Fig. 7C



BNSDOCID: <WO___9936103A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/00965

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 48/00; C12N 15/63; C12N 15/11; C07K 14/00; C07H 21/04

US CL :435/320.1; 514/44; 530/350; 536/23.1, 24.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1; 514/44; 530/350; 536/23.1, 24.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MAINA et al. Met receptor signaling is required for sensory nerve development and HGF promotes axonal growth and survival of sensory neurons. Genes and Development. 15 December 1997, Vol. 11, No. 24, pages 3341-3350, entire document.	1-23
Y	YAMAMOTO et al. Hepatocyte growth factor (HGF/SF) is a muscle-derived survival factor for a subpopulation of embryonic motoneurons. Development. 1997, Vol. 124, No. 15, pages 2903-2913, entire document.	1-23
Y	WONG et al. CNTF potentiates the effects of BDNF, GDNF, or HGF in cultured motor neurons. Society for Neuroscience Abstracts. November 1995, Vol. 21, Part 2, page 1535, entire document.	1-23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 26 FEBRUARY 1999	Date of mailing of the international search report 01 APR 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ANNE-MARIE BAKER, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/00965

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 24-31
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/00965

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GLOSTER et al. The Talpha1 alpha-tubulin promoter specifies gene expression as a function of neuronal growth and regeneration in transgenic mice. The Journal of Neuroscience. December 1994, Vol. 14, No. 12, pages 7319-7330, entire document.	1-23
Y	WU et al. Transcriptional repression of the growth-associated Talpha1 alpha-tubulin gene by target contact. J. of Neuroscience Research. 1997, Vol. 48, pages 477-487, entire document.	1-23
Y	HAMANOUE et al. Neurotrophic effect of hepatocyte growth factor on central nervous system neurons in vitro. J. of Neuroscience Research. 1996, Vol. 43, pages 554-564, entire document.	1-23

BNSDOCID: <WO___9936103A1_I_>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/00965

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS
DIALOG (file: medicine)
search terms: axon?, hepatocyte growth factor, neuron?, alpha tubulin promoter, neuropath?, diabet?, motor neuropath?, sensory neuropath?, sensorimotor

BNSDOCID: <WO___9936103A1_I_>